

The Role of Microtubule Retrograde Flow in Neuronal Development

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Abstract

The microtubule array in developing neurons consists of more than a hundred microtubules in each neurite. This microtubule array has been thought to act as stationary tracks, in which only individual microtubules are dynamic and could be transported. Using live cell imaging combined with photoconversion of microtubule patches I reveal that instead the whole microtubule array flows retrogradely into the soma. Before axon development, this microtubule retrograde flow (MT-RF) fuels the fluctuating state of neurons by decreasing microtubule density, leading to the long-known hallmark of microtubule density cycles instead of stable increases in microtubule density. MT-RF then slows down in the axon, right after initial axon formation, reducing microtubule density cycles to allow a more stable increase in microtubule density and thereby driving axon extension. I also observed that MT-RF later slows down in dendrites. By combining live-cell imaging with pharmacological and chemogenetic recruitment to the plasma membrane, I show that Dynein at the plasma membrane can fuel MT-RF. To understand the molecular effect of MT-RF on the microtubule array, I developed a biophysical model of microtubules in neurites across developmental stages and combined it with quantitative immunocytochemistry and microtubule dynamics data. Surprisingly, my model revealed that MT-RF slowdown is needed for both axon and dendrite development, uncovering a unified model of axon and dendrite development, in contrast to the prevailing idea that axon and dendrite development are triggered by different programs. In dendrites, my model and experiments show that MT-RF slowdown enables an efficient increase in stable microtubules. In axons, my model predicts that without slowdown of MT-RF,

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microtubules cannot reach close to the axon tip, blocking axon growth. MT-RF slowdown together with only three properties of the axonal and dendritic microtubule array, stability and two types of nucleation, is sufficient to explain the stable microtubule distribution in axons and dendrites, which drives neuronal polarization. In this thesis I found a novel type of cytoskeletal mechanism at an unprecedented scale in neurons and characterized its molecular function across developmental stages to find that it unifies the fluctuating state before axon development as well as axon and dendrite development. This provides new research avenues from the role of MT-RF in other processes and non-neuronal cell types to understanding how similar instead of different processes in axons and dendrites could underly neurodevelopmental .

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1. Introduction

1.1. Neuronal development

Neurons are complex computational cells that are integrated in networks to perform various behaviorally relevant computations¹⁻⁴ through their separation into an input- and output-region. Dendrites receive and integrate inputs that are processed and afterwards relayed via the axon through action potentials to the designated targets⁵⁻⁸. This difference between dendrites and the axon is key to neuronal function and is implemented at several levels, including the ultrastructure and molecular makeup. The specialization is evident through the distribution of proteins – with many proteins localizing exclusively to a specific compartment. But how does this strongly polarized structure emerge?

Studying neuronal polarization has been done for a long time, with the model of primary dissociated hippocampal neurons being the backbone of the research⁹⁻¹². Polarization in these neurons happens in five stages. Briefly after plating, these neurons form a lamellipodium (stage 1), from which soon after several small neurites grow (stage 2) (Figure 1). These neurites grow in short bursts but maintain similar lengths. Within 24 hours after plating, one neurite grows more than all the others, thereby becoming the axon, while the other neurites stay at the shorter length (stage 3) (Figure 1). Within the next week, the other neurites become dendrites (stage 4) (Figure 1). Eventually the axon further matures and specializes, forming branches and starting to form synapses¹³ (stage 5). Since this process happens in a cell culture dish, it does not need directional external cues which suggests that there is an intracellular machinery for polarization. This makes hippocampal neurons a great model for studying such intracellular mechanisms. Understanding these intracellular mechanisms of neuronal developmental - from initial axon growth to synapse development and synaptic transmission - is a promising avenue to develop new treatments for axonal regeneration¹⁴⁻¹⁹.

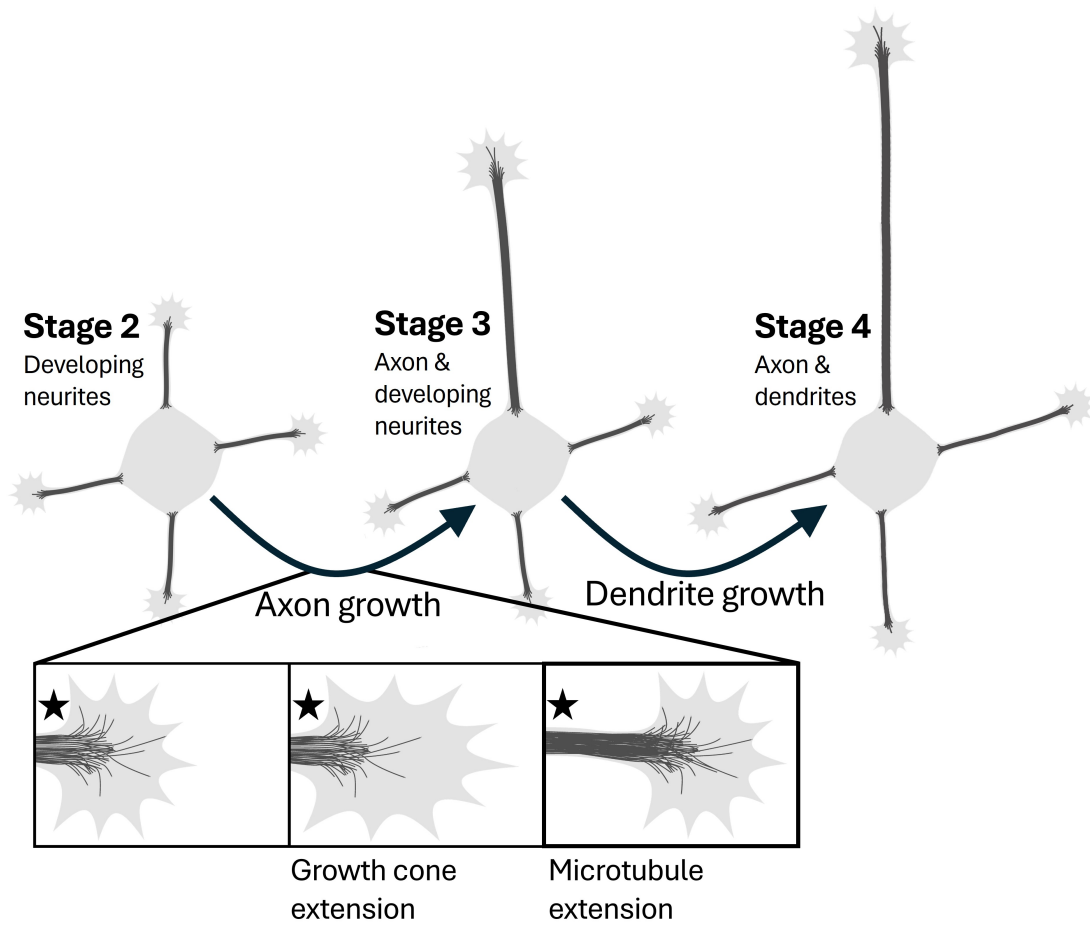


Figure 1. The stages of neuronal development focused on in this work.

In stage 2 the neuron has grown several developing neurites of about the same length. To transition into stage 3, one neurite grows to become the axon by first extending its growth cone and then also the MT array. Later the other neurites start growing more as well and become dendrites, which marks stage 4.

1.2. Neurite growth from the growth cone

One key mechanism for neuronal development is the growth of neurites. Without extensive neurite growth, the axon cannot develop²⁰. Neurites grow from their tips, that are specialized structures called growth cones (Figure 1). Growth cones are very dynamic, sensing and integrating several signals to guide the axon to its target. To regulate neurite growth in the growth cone, signals eventually converge on the actin and microtubule (MT) cytoskeleton²¹.

1.2.1. Actin

Actin is a highly abundant protein that can polymerize from globular actin (G-actin) monomers into dynamically growing and shrinking linear microfilaments (filamentous actin; F-Actin) to promote various types of cellular functions. Soluble actin is bound to adenosine triphosphate (ATP), which is hydrolyzed much more efficiently once actin is polymerized²². Once the phosphate dissociates from adenosine diphosphate (ADP)-actin, actin becomes more prone for depolymerization from F-Actin²². However, ADP-actin can also bind phosphate, which stabilizes its incorporation in F-Actin²³. This binding of phosphate is higher on one end of F-Actin, the barbed end, and lower on the other, so-called pointed end. Consequently, the barbed end of F-Actin usually incorporates more actin and thereby grows, while actin is depolymerizing from the pointed end, leading to shrinkage of F-Actin. This process of growth on one end and shrinkage on the other end is termed treadmilling and can keep an F-Actin polymer at the same length.

By interacting with other proteins, the dynamics of F-Actin are drastically changed. Profilin binds to monomeric actin and inhibits polymerization, allowing a much higher concentration of monomeric actin in cells than *in vitro*. On the other hand, formins²⁴ or Ena/VASP²⁵ can promote elongation. F-Actin can also be stabilized at both ends - with capping protein binding to the barbed end²⁵ and Tropomodulin binding to the pointed end²⁶. Nucleation of new F-Actin is promoted through formins²⁷ and actin-related protein 2/3 (Arp2/3)-mediated actin branching. Branched F-actin is a higher order organization of F-Actin which is essential for the formation of lamellipodia and thereby for the growth cone²¹. Through cross-linking, F-Actin can also form other higher order structures²⁸, particularly filopodia, which are the other prominent actin structure in growth cones. Lastly, actin filaments can also be severed, most commonly by proteins from the gelsolin family²⁹ or by cofilin^{30,31}. As outlined below, actin severing by cofilin is crucial for the growth cone³².

1.2.2. Microtubule dynamics

The other cytoskeletal structure crucial for neurons are MTs. MTs mainly act as railroads for intracellular transport but have also been shown to be involved in signaling³³ and generating forces, e.g. to lead to cell protrusions³⁴. They are hollow tubes with a diameter of 25 nm that are formed by heterodimers of α - and β -tubulin³³. MTs are highly dynamic and switch between growing and shrinking states, which is called dynamic instability³⁵(Figure 2), which is

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regulated by the binding state of tubulin. Cytosolic tubulin binds guanosine triphosphate (GTP) but this GTP is hydrolyzed soon after tubulin is incorporated into MTs. Since the resulting guanosine diphosphate (GDP)-tubulin destabilizes MTs, one prevailing hypothesis of MT growth is that a cap of GTP-tubulin is needed for stable growth. Once the cap is lost, MTs switch to the rapidly shrinking state called catastrophe (Figure 2). Interestingly, MTs are polarized structures, with dynamics being vastly different between the so called plus- and minus-end. The plus-end is much more dynamic, shows faster growth and switches more often to catastrophe than the minus-end³³. While classically only the plus-end was thought to grow, the minus-end was also shown to be able to grow in *Drosophila* neurons³⁶. Lastly, the generation of new MTs is usually regulated by nucleation via the γ -tubulin ring complex (γ -TuRC)³⁷ (explained in greater detail below in the sections “Centrosomal microtubule nucleation” and “Non-centrosomal microtubule nucleation”).

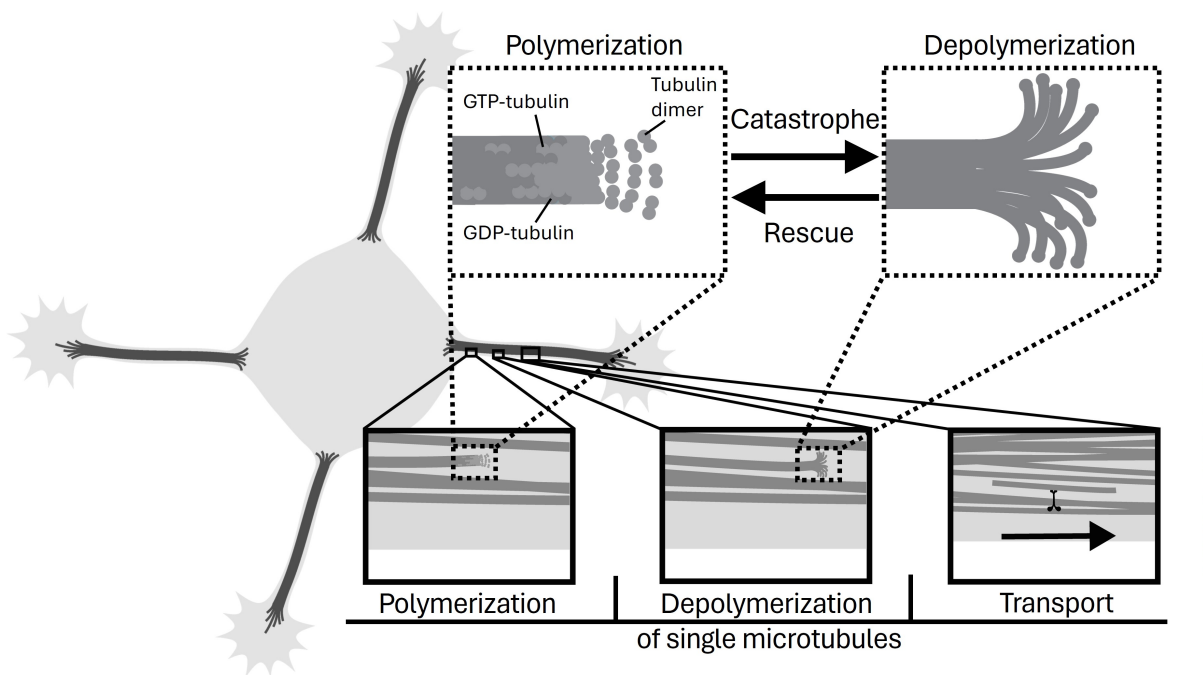


Figure 2. Single microtubule dynamics in neurons.

In neurons, MTs can grow (polymerization) or shrink (depolymerization) with small microtubules also being transported as a whole. For MT polymerization, GTP-tubulin heterodimers are incorporated into the MT. GTP-tubulin is then hydrolyzed to GDP-tubulin within the MT, which destabilizes MT polymerization. If the protective GTP-tubulin cap is lost, MTs quickly repolymerize (catastrophe), until they are either lost or transition back to the growing state through rescue.

1.2.3. Cytoskeleton in the growth cone

How are actin and MTs organized to form the growth cone? The growth cone is divided into three main compartments: the central domain (C-domain), the peripheral domain (P-domain) and the intermediary transition zone (T-zone)^{11,38,39} (Figure 3). The C-domain is filled with MTs that come from the neurite shaft (Figure 3) - these MTs can be bundled and have a higher fraction of stable MTs³⁹. On the other hand, the P-domain is defined by highly dynamic actin structures, particularly lamellipodia and filopodia and only contains few MTs³⁹ (Figure 3). P-domain MTs are very dynamic, as evident through their sensitivity to MT-depolymerizing drugs⁴⁰. Lastly, in the T-Zone condensed actomyosin structures, called actin arcs, are formed by myosin II mediated contraction of F-actin filaments³⁹ (Figure 3). Which dynamic actin and MT processes in the growth cone regulates its advance and thereby neurite growth?

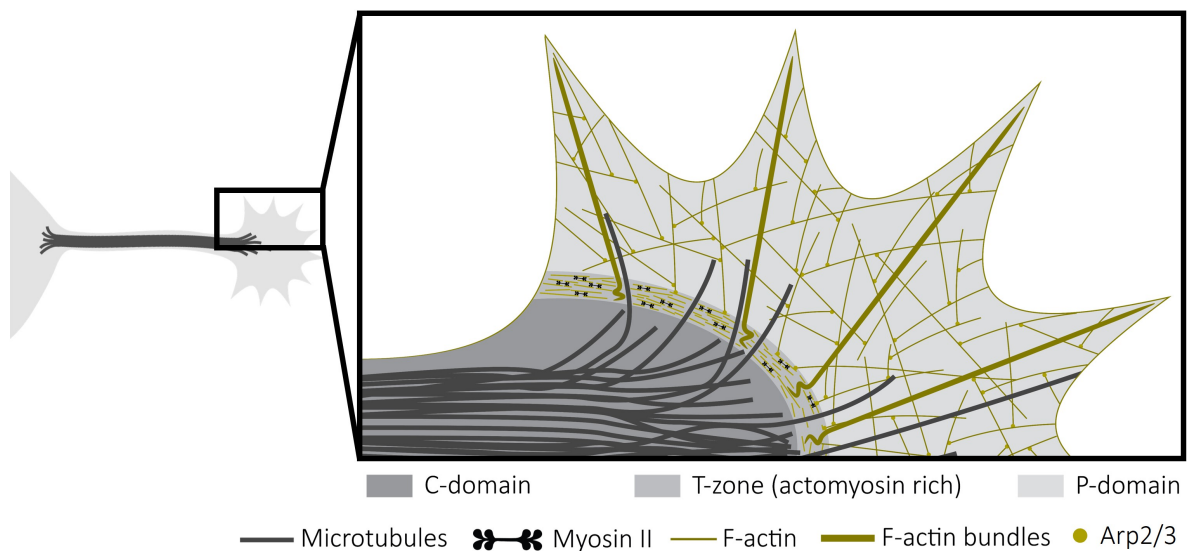


Figure 3. Cytoskeletal organization of the growth cone.

The neuronal growth cone is separated into three regions: the central domain (C-domain), the transition zone (T-zone) and the peripheral domain (P-domain). In the C-domain thick MT bundles can be found, which are hindered by the thick actomyosin network in the T-zone to enter the P-domain. Few MTs grow into the P-domain, where bundled actin networks and Arp2/3-mediated branched F-actin networks form filopodia and lamellipodia, respectively. The figure was adapted from Schelski and Bradke, 2017¹¹.

1.2.3.1. Actin retrograde flow

Actin that polymerizes in the P-domain of the growth-cone at the membrane generates a force towards the leading edge¹¹. Because of membrane tension⁴¹, the polymerizing actin does not deform the membrane, leading to a retrogradely-directed force that moves actin backwards towards the T-domain. Additionally, in the T-domain, myosin II contracts actin and thereby generates a pulling force that pushes retrogradely against the actin cytoskeleton, also moving actin retrogradely. The combined retrogradely-directed forces of peripheral actin polymerization and pulling of actomyosin result in a retrograde flow that moves F-actin into the T-zone^{39,42}. To make space for new F-actin in the T-zone, actin filaments are severed into smaller fragments by Cofilin³². This severing also provides G-actin for actin polymerization at the leading edge³⁹. This actin polymerization at the leading edge can lead to membrane protrusions that extend the growth cone.

1.2.3.2. Regulating actin-mediated protrusion in the growth cone

Actin-mediated protrusions are important to extend the growth-cone's leading edge and therefore are thought to enable neurite growth¹¹. The size of the protrusion is a result of the balance of membrane tension and the membrane-directed force. As a consequence, a lower membrane tension, induced by membrane insertion, or enhancing the pushing forces, increases protrusion size⁴¹. Pushing forces in the growth cone are caused by F-actin polymerizing against the leading edge and can thus be amplified by increasing the number of actin filaments pushing against the membrane. Rac1 is a key regulator of this process in neuronal polarization⁴³. Rac1 is a Ras homologous (Rho) GTPase, which is active when bound to GTP; when GTP is hydrolyzed to GDP, Rac1 is inactivated⁴⁴. To activate GDP-bound Rac1, GDP has to be exchanged for GTP, which is promoted by guanine nucleotide exchange factors (GEFs). Overexpressing Rac1 or its GEF T-lymphoma invasion and metastasis-inducing protein 1 (Tiam1), increases neurite growth and leads to the formation of multiple axons^{45,46}. On the other hand, suppressing Tiam1 reduces the increased actin dynamics that are characteristic for axonal growth cones^{45,47}.

Rac1 regulates actin dynamics primarily by activating the Wiskott-Aldrich syndrome protein (WASP)-family vevprolin-homologous protein (WAVE)⁴³. WAVE in turn enables actin branching through Arp2/3, inducing lamellipodia^{48–52}. These lamellipodia enhance protrusions, enabled through recruiting WAVE and Arp2/3 to the leading edge of the growth cone⁵³.

Conversely, inhibiting Arp2/3 reduces free barbed F-actin ends and reduces nerve growth factor (NGF)-mediated F-actin increase⁵³. While NGF still leads to protrusions during Arp2/3 inhibition, these protrusions are unstable⁵³. This instability shows that a strong force is necessary to overcome membrane tension to lead to protrusions⁴¹ and consequently reducing the number of actin filaments that push against the membrane probably destabilizes protrusions. All in all, Rac1 increases the F-actin pushing force directed against the membrane and thereby can increase membrane protrusion.

1.2.3.3. Moving the growth cone forward: clutch or amoeboid?

One way how the growth cone was thought to move forward was by myosin II-mediated forces, pulling F-actin retrogradely, being translated to pulling the growth cone forward¹¹. By binding F-actin to the extracellular matrix a stable anchor was thought to be formed based on which force can be exerted. This process is known as the “clutch hypothesis”. F-actin can be coupled to the extracellular matrix by promoting the binding of adhesion sites to F-actin, e.g. through the action of Shootin1⁵⁴. This is regulated by Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42), which activate p21-activated kinase 1 (PAK1), in turn phosphorylating and thereby activating Shootin1^{54,55}. Phosphorylated shootin1 interacts with the L1 Cell Adhesion Molecule (L1-CAM) and binds to F-actin through cortactin^{54,55}. This L1-shootin1-cortactin complex acts as a molecular clutch that allows myosin II to generate the force that pulls the growth cone forward^{54,55}. The coupling of actin to the extracellular matrix reduces retrograde movement of F-actin from the leading edge, by acting as a stable connection point resisting the retrograde force. This enhances the force of actin polymerization directed outwards, leading to actin protrusions and thus growth cone enlargement.

All of this would suggest that myosin II should be important for neurite growth. Instead, myosin II inhibition increases neurite growth and induces the formation of multiple axons^{56,57}. Additionally, the clutch hypothesis suggests that axonal growth cones should pull on the extracellular matrix to move forward. However, recently it was shown that axons of hippocampal neurons in a 3-dimensional (3D) collagen matrix do not pull on the extracellular matrix, in contrast to fibroblasts⁵⁸. This shows that central nervous system neurons in fact do not move forward by pulling on the extracellular matrix. Instead, it was suggested that neurons move their growth cone forward in an amoeboid fashion that is independent of adhesions⁵⁸.

This suggests that not myosin and actin but instead potentially MTs in the growth cone are crucial for the forward movement of the growth cone.

1.2.3.4. Microtubules in the growth cone

Most MTs only reach until the C-domain of the growth cone with only a few ones extending into the P-domain (Figure 3). The extension of MTs into the P-domain and particularly to the leading edge is an important regulator of neurite growth¹¹. These MTs can allow vesicle transport to the leading edge and in turn membrane insertion. This reduces membrane tension and thereby allows actin to deform the membrane to fuel neurite growth. The process of MTs extending into the P-domain is inhibited by the dense actomyosin mesh in the T-domain⁵⁹. Thus, MT-mediated neurite growth is thought to be mainly regulated through changing the T-domain barrier to allow more or fewer MTs to extend into the P-domain. Importantly, stabilizing MTs locally in the growth cone of developing neurites, increases the chance of this neurite becoming the axon, while stabilizing MTs with taxol in the entire neuron leads to the formation of multiple axons⁶⁰. This supports the crucial role of MTs for neurite growth and axon development.

While a lot of research has focused on how processes at the growth cone enable neurite growth, processes in the neurite shaft are also known to be key to neuronal polarization by enabling cargo sorting and thereby molecular polarization.

1.3. Microtubules in the neurite shaft

Due to their extended morphology, neurons need to supply a much larger distance with cellular material than other cells. This makes MTs, the railroads of the cell, particularly important for neurons. The entire axon and dendrites are filled with complex networks of ~ 10-100 parallel MTs⁶¹ to allow the necessary efficient transport of proteins and vesicles. To form these MT networks, the MT dynamics described above can be modified by a wide range of different proteins. Proteins can target MT ends to regulate their dynamics, either plus-end targeting proteins (+TIPs) or minus-end targeting proteins⁶², they can regulate the nucleation of new MTs³⁷ or sever existing MTs⁶³. Additionally, MTs can be post-translationally modified to change their interaction with other proteins⁶⁴ and proteins can bind to the lattice of MTs to stabilize them⁶⁵. Interestingly, not only is the morphology of neurons polarized, but the MT networks in dendrites and axons are polarized as well. While there are more MTs in the young

axon than in the other neurites^{66,67}, MT orientation and stabilization also differ and change within each neurite during development. This polarized MT network is the basis for polarized transport and therefore thought to be key for molecular and morphological neuronal polarization⁶⁸. And how does a polarized MT network enable morphological polarization (MT based transport) and how do these polarized MT networks in axons and dendrites form (MT orientation, MT stability, MT nucleation)? And how is the MT array in neurites regulated (Transport of MTs, MT severing)?

1.3.1. Microtubule-based transport

MT-based transport is the main mechanism by which MTs drive the molecular and morphological polarization of neurons. MT-based transport is required for many functions in neurons and to allow the soma to signal to synapses as well as the synapses transmit signals to the soma efficiently^{61,69,70}. A vast number of cellular components are transported in neurons, including mRNAs, receptors, organelles and precursors of synaptic vesicles^{69,71}. To enable molecular polarization, transport is polarized as well. Cargo sorting is enabled through local signaling and proteins that act as cargo-adaptors which regulate cargo loading and cargo movement⁷⁰. As a consequence of the polarity of single MTs, transport is polarized as well – with most kinesins mediating plus-end-directed transport and dyneins as well as some kinesins minus-end-directed transport (Figure 4).

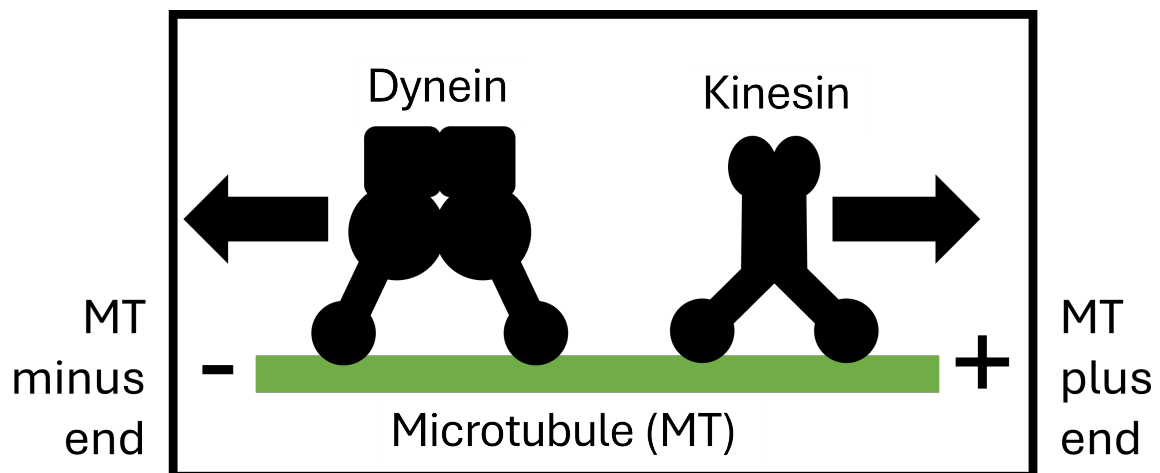


Figure 4. Transport on microtubules by dynein and kinesin.

Transport on MTs is directional, with dynein moving towards the MT minus-end and most kinesins (N-kinesins, specifically) move towards the MT plus-end.

1.3.1.1. Kinesin-based transport

Many proteins are part of the kinesin superfamily and can be sorted into three groups. In total 45 kinesin superfamily (KIF) proteins have been identified, separated into groups of 14 classes⁷². They are defined by the motor domain position in the protein: C-terminal motor domain KIFs (C-KIFs), middle motor domain KIFs (M-KIFs) and N-terminal motor domain KIFs (N-KIFs)⁶⁹. Only C-KIFs and N-KIFs are responsible for the transport on MTs with the last group, M-KIFs, depolymerizing MTs. The most common, N-KIFs, enable plus-end-directed transport while the smaller group of C-KIFs moves towards the minus-end of MTs^{69,73}. While most kinesins form homodimers (e.g. KIF5) or heterodimers (e.g. KIF3), some kinesins act as monomers (e.g. KIF1A)⁶⁹. In order to move along MTs, the kinesin motor domain binds MTs and hydrolyzes ATP. The end of the kinesin that does not contain the motor domain typically binds the cargo⁷⁴.

1.3.1.2. Dynein-based transport

The dynein superfamily mainly consists of two groups, axonemal dynein and cytoplasmic dynein. Only cytoplasmic dynein is responsible for intracellular transport and is made up of several proteins coming together in one big protein complex⁶⁹. This cytoplasmic dynein protein complex is formed by two heavy chains, that contain the motor domain, two intermediate chains, four intermediate light chains and additional light chains^{75,76}. Importantly, Dynein activity and cargo binding is regulated by the crucial accessory complex dynactin that itself is formed by several different proteins⁷⁷. While the kinesin superfamily contains 45 KIFs, there are only two proteins of the dynein heavy chain family, cytoplasmic dynein heavy chain 1 (Dync1h1) and cytoplasmic dynein heavy chain 2 (Dync2h1)^{69,78,79}. Interestingly, these two heavy chains serve different functions, with mainly Dync1h1 being responsible for cytoplasmic transport and Dync2h1 mainly taking care of intraflagellar transport⁸⁰. Despite only one dynein heavy chain taking care of cytoplasmic transport, dynein is still functionally diverse, through diversity in the several chains of the dynein complex and in dynactin⁶⁹.

1.3.2. Transport of microtubules

While MTs are the railroads on which other proteins are transported, MTs can also be transported themselves. Particularly the transport of short MTs has been observed in the axon and is thought to be a source of seeds for new MTs^{81–83}. For MT transport early in development,

when the centrosome is not yet switched off^{84,85}, MTs are nucleated at the centrosome, then severed and subsequently transported into neurites and young axon^{83,86}. However, since switching off the centrosome early in development does not affect axon development and growth, transport of centrosome-nucleated MTs seems to not be crucial. Nonetheless, MT transport could help supply MTs deeper into the neurite and axon, without the need for these MTs to be centrosomally nucleated. Additionally, transport of MTs is one of the mechanisms enabling the polarized MT orientations in axons and dendrites (see section “Microtubule orientation”).

1.3.3. Post-translational modifications of tubulin

In order to allow polarized transport into axons and dendrites, MTs are post-translationally modified to direct transport. Various post-translational modifications of tubulin act as cues to determine the interaction of MTs with other proteins⁶¹. The carboxy-terminal tails are modified through the action of many enzymes that act on tubulin incorporated into MTs^{64,87}. The opposite reactions are catalyzed on free tubulin, keeping newly incorporated tubulin without modifications. Common modifications are acetylation, polyglycylation, phosphorylation, polyglutamylation and the cleavage of the tyrosin at the C-terminus (detyrosination)⁸⁸. Since these modifications happen specifically on tubulin incorporated in MTs, longer lived MTs accumulate more modifications. Therefore, tubulin modifications, like acetylation and detyrosination, are well established markers for stable MTs in neurons^{60,87,89–91}.

Before axon development, single neurites show increased levels of acetylation and detyrosination compared to the other neurites⁶⁰. The levels of acetylation and detyrosination then further increase in the axon^{60,89}. This indicates that single neurites before axon development have increased levels of stable MTs and that stable MTs further increase in axons. These differences in post-translational modifications of tubulin increase transport into the axon due to modulating the affinity of some kinesins to MTs. The overexpressed motor domain of KIF5C (kinesin-1) accumulates specifically in the tip of single neurites before axon development and later in the tip of the axon⁹². This accumulation was shown to be driven by post-translational modifications. Indeed, taxol treatment, which stabilizes MTs and increases multiple post-translational modifications, leads to kinesin-1 accumulation in all neurite tips⁶⁰.

But which post-translation modification is responsible to drive kinesin-1 into the axon? Kinesin-1 binds more strongly to acetylated MTs⁸⁹ and GTP-tubulin⁹³, both more common in

the axon^{60,89,93}. However, increasing acetylation by inhibiting deacetylation through histone deacetylase inhibitors does not noticeably change kinesin-1 localization. In addition to acetylation and GTP-tubulin increasing kinesin-1 binding, tyrosination of MTs was shown to inhibit kinesin-1⁹⁴. Importantly, increasing detyrosination is sufficient to direct kinesin-1 to all neurites⁹⁴, showing that kinesin-1 accumulation is driven primarily by detyrosination of MTs.

Interestingly, not all kinesins are modulated by these post-translational modifications. In contrast to kinesin-1, the KIF1a motor domain accumulates in all neurite tips equally⁹². This allows post-translational modifications of MTs to serve a more complex role than only increasing transport into the axon. Since these transport dynamics are the basis for the molecular polarization of neurons, understanding how post-translational modifications emerge and polarize is crucial. Since post-translational modifications accumulate more on longer lived, stable MTs, unstable and stable MTs can show very different modifications. This raises the question of which mechanisms determine the stability of MTs.

1.3.4. Stabilization of microtubules

MTs can be stabilized by at least two basic mechanisms. First, their polymerization dynamics can be changed by influencing the plus-end or by changing the supply of tubulin dimers. And second, the MT lattice can be stabilized, e.g. through binding of MT associated proteins (MAPs) or the insertion of GTP-islands^{95–97}.

These mechanisms to stabilize MTs are regulated by many signaling pathways in neurons. Rac1, while classically known for its role in regulating branched actin⁴³, also affects MTs during neuronal development⁹⁸. Dedicator of Cytokinesis 7 (DOCK7) activates Rac1 to in turn stabilize MTs by inactivating stathmin, which sequesters tubulin dimers and thereby destabilizes MTs^{99–101}.

A well-known upstream regulator of MT dynamics is phosphoinositide 3-kinase (PI3K). PI3K regulates MT dynamics by activating glycogen synthase kinase-3 β (GSK3 β) through interleukin-like kinase (ILK) and protein kinase B (Akt). This activates several MAPs by promoting their dephosphorylation: Tau, cytoplasmic linker associated protein 2 (CLASP2), adenomatous polyposis coli (APC) and collapsing response mediator protein 2 (CRMP2)^{102–108}. GSK3 β also activates MAP1B, but by phosphorylation it^{109,110}. GSK3 β -mediated mechanisms are well known to stabilize MTs in the growth cone.

MAP1B enhances MT polymerization while keeping dynamic instability intact^{111–113}. When MAP1B is dephosphorylated, it binds and sequesters end binding protein 1 (EB1) and end binding protein 3 (EB3) in the cytosol which reduces MT growth¹¹⁴. Once MAP1B is phosphorylated, EB1 and EB3 are freed to in turn bind to the dynamic MT plus-end and increase MT polymerization¹¹⁴. The association of MAP1B primarily with the dynamic MT plus-end is further supported by its localization to dynamic, tyrosinated MTs, further indicating that a key role of MAP1B is to keep MTs in the P-domain dynamic^{113–115}. In neuroblastoma cells, MAP1B was also shown to promote MT bundling¹¹⁶.

CRMP2 stabilizes heterodimers of tubulin and thereby increases MT polymerization (Fukata et al., 2002). Locally inhibiting CRMP2 in turn leads to growth cone turning¹¹⁷. Tau, another MAP affected by GSK3 β , makes MTs more rigid and increases MT bundling^{118,119}. Tau is one of the MAPs that binds to the MT lattice, particularly of dynamic MTs in the P-domain and is responsible for Wnt5a-induced turning of the growth cone^{120–122}.

Apart from growth cone turning, GSK3 β pathways is also important for neuronal polarization. GSK3 β -mediated CRMP2 activity is driving neuronal polarity^{106,123}. The role of Tau¹²⁴, CLASP2¹²⁵, and MAP1B^{124,126} in neuronal polarity is also clear, but how much this role in physiology is mediated by GSK3 β is not known. Additional proteins like MT-affinity regulating kinase 2 (MARK2) and the liver kinase B1 (LKB1) - synapses of amphids defective (SAD) pathway have important roles in neuronal polarity and also interact with MAPs^{127–130}. The plus-end-binding proteins cytoplasmic linker protein (CLIP) - associating protein (CLASP) are also known to stabilize MTs. CLASPs increase MT polymerization through binding the plus-end of MTs and increasing rescues³³. In neurons CLASPs affect particularly neuronal polarity and axonal growth^{125,131}. Despite the wealth of pathways known to influence MT stability we still only poorly understand what drives increased MT stability in young axons and the role of MT stabilization in dendrites.

1.3.5. Microtubule severing

Seemingly paradoxically, MT severing enzymes can also stabilize MTs, through GTP-insertion. MTs can be cut by specific enzymes, either from the spastin, katanin or fidgetin protein class^{61,63,132}. All these proteins are highly expressed in neurons and influence neuronal physiology. The actions of katanin and spastin induce axonal branching and are also important in dendrites during development¹³³ while fidgetin has been mostly implicated in reducing axon

growth¹³⁴. MT severing exposes the minus-end of MTs and a new plus-end in the middle of the MT. The minus-end is inherently unstable and will depolymerize without stabilization. But even the fresh plus-end tends to be unstable, since it is often not protected by a GTP-cap. This could lead to the conclusion that MT severing is a mechanism to reduce MTs.

However, both the minus-end and the lattice at which MTs can be cut are highly regulated in neurons and can be stabilized. The fresh plus-end often is actually not unstable. In stable MTs the lattice itself is stabilized, possibly also through in-lattice MT repair with GTP-tubulin⁹⁷, which prevents catastrophe of the fresh plus-end¹³⁵. Additionally, MT severing enzymes themselves can insert small GTP islands into the MTs that support MT growth⁹⁶. The minus-end on the other hand side can be stabilized by minus-end binding proteins, most prominently calmodulin-regulated spectrin-associated proteins (CAMSAPs)¹³⁶. This leads to the formation of a stable seed for MTs. By these mechanisms, katanin and spastin can stabilize cut MTs to increase the number of growing MTs and thereby increase MT mass^{96,137}, while fidgetin in contrast reduces MT mass¹³⁴. Therefore, MT severing in neurons can be an important mechanism to increase MT mass, depending on the MT severing enzyme, due to the formation of a new plus-end from which the MT can polymerize.

1.3.6. Microtubule orientation in neurons

Based on these transport mechanisms, how are molecular and morphological polarization enabled by MT-based transport? The key to driving polarized transport into the axon and dendrites is the difference in MT orientation in these two compartments. While >95% of MTs in the axon are oriented with their plus-end towards the axon tip (plus-end-out)^{138,139}, in dendrites equal proportions of MTs are plus-end-out and with their plus-end oriented towards the soma (plus-end-in)¹³⁹ (Figure 5). In young neurons without an axon (stage 2), MTs are oriented bidirectionally, with 80% of MTs being in the plus-end-out orientation¹³⁹.

Enriching the proportion of plus-end-out MTs for the axon is crucial for increasing anterograde transport into the axon. On the other hand, in dendrites, mixed MT orientation is critical for correct cargo sorting^{140,141}. While plus-end-out MTs are preferentially unstable in dendrites, plus-end-in MTs are preferentially stable¹⁴¹. This results in post-translational modifications being differentially distributed on MTs of different orientations – with plus-end-out MTs being preferentially tyrosinated and plus-end-in MTs preferentially detyrosinated and acetylated¹⁴¹. Axon-specific cargo transported by kinesin-1, which is preferentially transported

on detyrosinated MTs⁹⁴ (see section “Post-translational modifications of tubulin”) would in turn be transported out of the dendrite through plus-end-in oriented MTs, while other kinesins and especially dynein could transport non-axonal cargo into the dendrite¹⁴⁰.

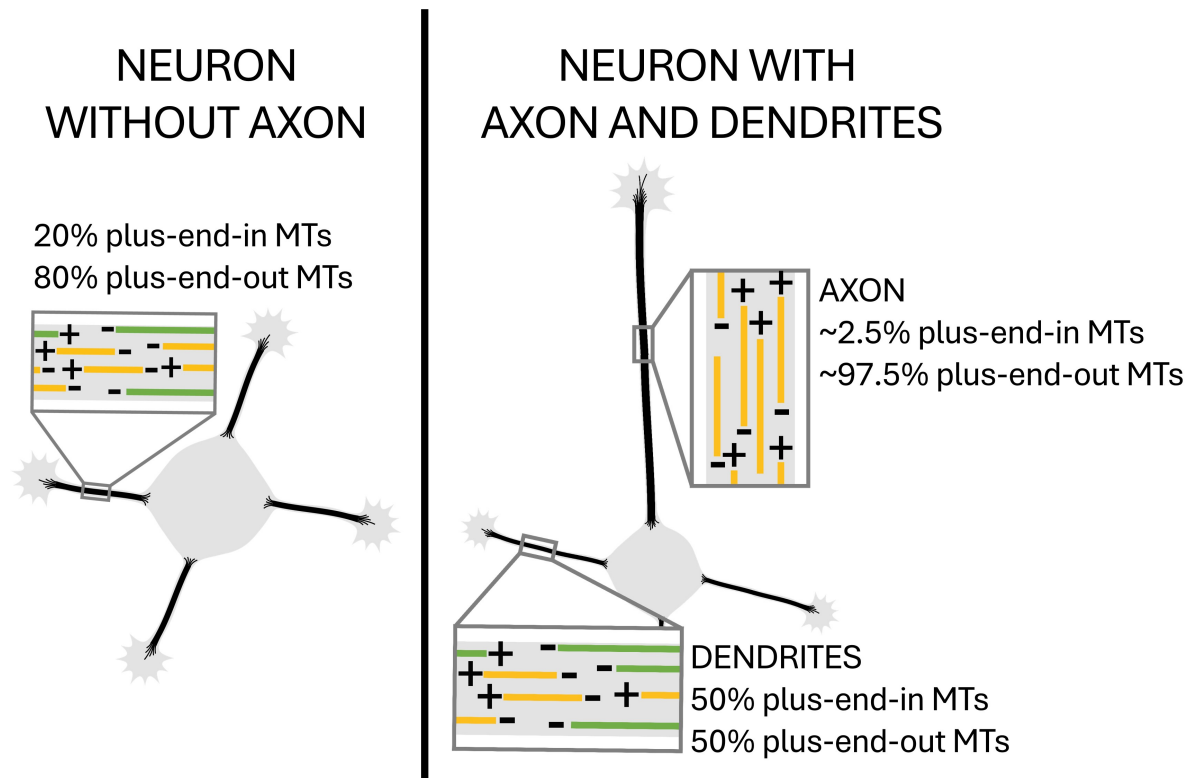


Figure 5. Microtubule orientation in developing neurites, the axon and dendrites.

Before axon development, MT orientation in developing neurites is mixed, with most (80%) of MTs being plus-end-out oriented and the rest (20%) being plus-end-in oriented. After the axon and dendrites have developed, MT orientation is highly polarized between the two compartments. While almost all MTs (~97.5%) are plus-end-out oriented in the axon, in the dendrite only roughly 50% of MTs are plus-end-out oriented. The illustration of the neuron with axon and dendrites is adapted from Schelski and Bradke, 2022¹⁴².

But which mechanisms fuel the polarization of MT orientation in axons and dendrites? In axons, plus-end-out MTs were shown to passively accumulate due to the increased axonal length, the key hallmark of axons, which favors MTs growing towards the axon tip⁶⁷. However, this mechanism by itself could not explain the very high proportion of plus-end-out MTs in axons. Plus-end-in MTs are also transported out of the axon through dynein-mediated transport, possibly from actin filaments as a stable basis^{143–146}. An additional mechanism to increase plus-

end-out MTs in the axon is mediated by tripartite motif containing 46 (TRIM46), which bundles and stabilizes specifically MTs of the same orientation¹⁴⁷. Importantly, TRIM46 accumulates close to the base of the axon at the soma, at the future axon initial segment, and is thought to act as a gateway for unipolar MTs¹⁴⁸. Indeed, TRIM46 knockdown inhibits axon development¹⁴⁸. Another proposed mechanism is the accumulation of plus-end-out MTs due to increased growth of MTs close to the axon tip¹⁴⁹. This increased growth is specific to plus-end-out MTs and in *Drosophila* is mediated by a gradient of a protein inhibiting MT catastrophe from the growth cone¹⁴⁹. Lastly, MT nucleation also plays a crucial role in maintaining uniform plus-end-out MT orientation in axons, through reducing the nucleation of plus-end-in MTs¹⁵⁰, as explained in more detail below (“Non-centrosomal microtubule nucleation”).

In dendrites, mixed MT orientation was proposed to stem from MTs that are severed from the centrosome and then reorient through sliding by motor proteins^{81,86,151,152}. The kinesins kinesin-6 and kinesin-12 were implicated in increasing minus-end-out MTs, through transporting MTs oriented with their minus-end-out on plus-end-out oriented MTs^{153,154}. In summary, a combination of different MT transport and MT nucleation form the uniform plus-end-out MT orientation in axons and the mixed MT orientation in dendrites.

1.3.7. Centrosomal microtubule nucleation

How are new MTs generated in neurons? MTs do not generate spontaneously from tubulin but instead the mechanisms generating new MTs are tightly regulated. New MTs can be nucleated from different MT organizing centers (MTOCs), which provide an anchor for the minus-ends of MTs^{37,155}. In young neurons, many MTs are nucleated at the centrosome, the complex MT organizing center (MTOC) enabling chromosome separation during cell division^{85,86}. The centrosome is made up of two centrioles, which recruit pericentriolar material (PCM), including the γ -tubulin ring complex, a critical complex for MT nucleation¹⁵⁵.

MTs nucleated at the centrosome are insufficient by themselves for the extended neuronal morphology, as they would not reach far into the neurites^{156–158}. One mechanism to allow centrosomally nucleated MTs to get further into the neurites is severing from the centrosome and subsequent transported into neurites¹⁵⁹. However, the activity of the centrosome is steadily reduced during neuronal polarization and after a few days barely any MTs are nucleated at the centrosome^{84,85}. Consequently, removing or switching off the centrosome does not affect axon

growth⁸⁴ and neuronal polarization⁸⁵. In more mature neurons other means of nucleating MTs are important to supply MTs throughout their extended morphology.

1.3.8. Non-centrosomal microtubule nucleation

Neurons generate new MTs needed for their long axon and dendrites through various non-centrosomal mechanisms³⁷. The type of nucleation determines the orientation of the new MT and thereby regulating MT nucleation is key to the uniform MT orientation in axons¹⁵⁰.

First, MTs can be nucleated from the Golgi apparatus in the soma and might be nucleated from Golgi outpost throughout the neuron³⁷. MTs growing from the soma into the axon would grow with their plus-tip towards the axon, these MTs would be plus-end-out oriented (Figure 6).

Additionally, MTs can act as the basis for nucleation themselves through branching nucleation of MTs. Augmin and the HAUS Augmin Like Complex Subunit 1 (HAUS) protein complex are key regulators of branching nucleation in neurons^{150,160}. They recruit γ -tubulin ring complex (γ -TuRC) to the MT lattice to allow MT nucleation. Since augmin knockdown also reduces MT bundles in the soma¹⁵⁰, the shallow branching angle could be a mechanism to promote the formation of bundled MTs^{161,162}. Importantly, the MT branch grows at a defined range of shallow angles that lead to the same MT orientation of the newly formed MT as the MT it is nucleated on^{161,163,164} (Figure 6).

As mentioned above (section “Microtubule orientation”), non-centrosomal MT nucleation is important for uniform plus-end-out MT orientation in the axon. Importantly, knockdown of augmin reduces the fraction of plus-end-out MTs in axons noticeably¹⁵⁰. This effect depended on γ -Tubulin, while expressing a nucleation-activated form of γ -Tubulin by itself reduced the plus-end-out fraction¹⁵⁰. Thus, it was proposed that augmin-mediated MT branching competes with a form of non-centrosomal MT nucleation in the cytosol¹⁵⁰.

Indeed, MTs in neurons are also generated from many different spots in the cytosol, as evident from MT regrowth after nocodazole-mediated MT depolymerization¹⁶⁵. This cytosolic MT nucleation was also associated with the presence of γ -TuRC¹⁵⁰, supporting the idea this MT nucleation competes with augmin-mediated MT branching. Since cytosolic MT nucleation should lead to equal proportions of plus-end-out and plus-end-in MT orientation (Figure 6), a reduced competition with augmin, through augmin knockdown, would increase the fraction of plus-end-in MTs in axons, as reported¹⁵⁰. This suggests that augmin mediated MT branching

drives the uniform plus-end-out MT orientation in the axon through suppressing cytosolic MT nucleation.

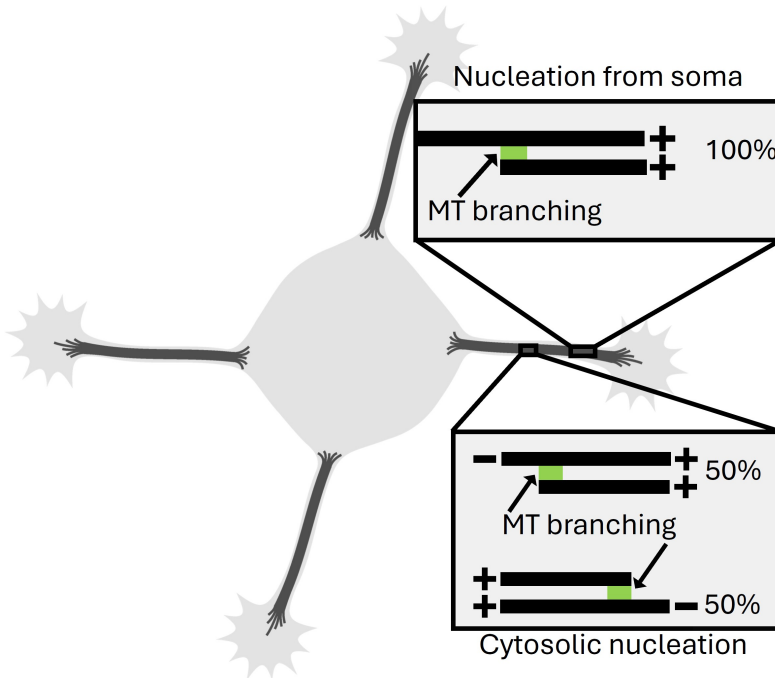


Figure 6. The effect of MT nucleation on MT orientation.

MTs nucleating in the soma and growing into the neurite are plus-end-out oriented. On the other hand, MTs nucleating in the cytosol have an equal chance of being plus-end-out or plus-end-in oriented. MTs formed by augmin-mediated MT branching have the same orientation as the MT they are nucleated from. Parts of the figure are adapted from Schelski and Bradke, 2022¹⁴².

1.4. The fluctuating state before axon development

But how and when are MT stabilization and MT orientation orchestrated to develop the polarized MT network in axons and dendrites? In fact, not only stable MTs polarize before axon development, as mentioned earlier, but the entire MT network already polarizes before axon development (Figure 7). However, this polarization is only transient and highly dynamic before axon development. Before axon development, neurons are in a fluctuating state in which single neurites grow, before they retract again to maintain a relatively constant length¹⁶⁶. This growing state fluctuates stochastically between neurites (Figure 7). When a neurite enters this growing state, it obtains several axon-like properties, which are thought to enable its growth. At the point when the neurite retracts, these axon-like properties are lost again.

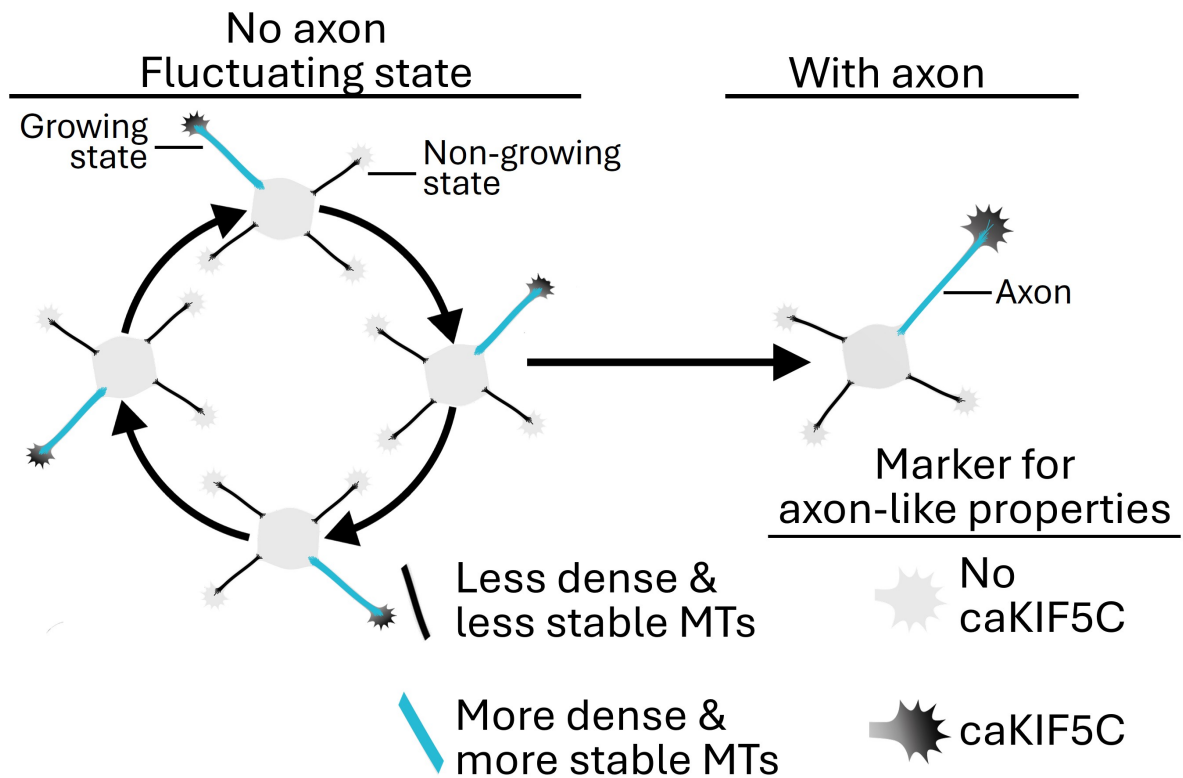


Figure 7. The fluctuating state before axon development and its consolidation in the axon.

Before axon development, neurons are in a fluctuating state, in which single neurites grow and retract again without gaining a lot of length. While neurites are growing, they transiently increase their MT mass, the fraction of stable MTs and they accumulate the kinesin-1 motor (caKIF5C) in their growth cone. These axon-like properties are lost again when the neurite retracts. When the axon develops, all properties are stably consolidated in the axon. Figure adapted from Schelski and Bradke, 2022¹⁴².

The trigger for gaining axon-like properties is thought to be a wave of polymerizing actin (actin wave) that is generated in the soma and travels into a neurite^{56,167}. Alongside the actin wave, the kinesin-1 motor moves into the neurite up to the growth cone^{92,168} (Figure 7). Kinesin-1 is directed to neurites through increased dephosphorylation of MTs and thereby increased stable MTs⁹⁴. This indicates that MT stability is also stochastically increased in single neurites^{60,89} (Figure 7).

Within and at the back of the actin wave, more MTs are growing, as indicated by an increased number of EB3 comets¹⁶⁸, which leads to an increased MT density in the neurite (Figure 7). However, whether the increased number of EB3 comets comes from increased MT nucleation, increased rescue, reduced catastrophe or another mechanism remains unclear.

But not only MT stability and density polarize before axon development. Recently, single neurites were also shown to have a uniformly plus-end-out oriented MT array before axon development⁸⁶. This suggests that generating the uniform MT orientation array is also a very early step in axon development and could fluctuate between neurites before axon development. This uniform MT orientation could drive axon-like cargo into transiently polarized neurites. Interestingly, in developing neurites with mixed MT orientation, stable MTs are already preferentially plus-end-in oriented⁸⁶, which could keep axonal cargo preferentially out of non-polarized developing neurites, as it does in dendrites¹⁴¹. However, the stable MT fraction of plus-end-in MTs is less compared to dendrites, suggesting that axonal cargo is kept out of developing neurites less efficiently than out of dendrites⁸⁶.

Eventually, axon-like properties are consolidated by one neurite stochastically growing enough to form the axon (Figure 7). The length dependent consolidation of the axon is thought to be conferred by reduced diffusion through the longer axon. This leads to inhibitory, diffusive signals from the soma going less into the axon¹⁶⁹ and axonal cargo accumulating more in the axonal tip due to reduced diffusion out of the axon^{11,170–174}.

1.5. Mathematical models of microtubules in neurons

MT dynamics have been modeled in vitro to understand the properties of dynamic instability^{175,176}. These models were used particularly fruitfully to understand cell division, where the role of MT-related forces, MT flux and MT sliding for chromosome positioning and segregation have been investigated^{177–180}. In neurons, a few mathematical models investigated the emergence of uniform MT orientation in axons^{145,149} and the increase in MT mass of axons⁶⁷. However, none of these models considered MT stabilization, a key property which needs to change for the fluctuating state, as well as axon development. Thus, it remains unclear how stable MTs emerge in neurons during different developmental stages; a unifying model of neuronal development is lacking.

1.6. Aims of the thesis

It is still unclear how the entire neuron can stay in the fluctuating state before axon development, specifically we don't know why a neurite loses axon-like properties after some time. In this work we want to understand how the fluctuating state before axon development is maintained and whether there are other mechanisms than neurite growth to stabilize axon

identity. We further want to understand how dendrite development is triggered and which mechanisms activate the growth of mature dendrites, considering that developing neurites do not show steady growth. To answer these questions, we looked at neurite shaft MTs, since their role beyond enabling polarized transport has remained elusive. Neurite shaft MTs are the basis for the accumulation of axon-like properties in single neurites and therefore for the fluctuating state. But why do shaft MTs stay in an axon-like state only transiently? And how are dendrite and axon growth connected? Even though the growth cone of axons and dendrites differs in several aspects, both growth cones have in common that they need MTs to enable growth. Could dendrite growth therefore somehow be triggered by the same mechanism that triggers axon growth?

I found a previously unknown type of MT dynamics, the movement of the entire MT array towards the soma retrogradely. I found that this MT retrograde flow (MT-RF) is needed to maintain the fluctuating state and that its slowdown enables both axon and dendrite development. I present a unifying model on how neuronal development is executed across developmental stages.

1. Introduction

2. Microtubule retrograde flow retains neuronal polarization in a fluctuating state

This chapter of the thesis represents a paper that was published 4th of November 2022 by the American Association for the Advancement of Science in the journal *Science Advances*¹⁴².

Schelski, M., & Bradke, F. (2022). Microtubule retrograde flow retains neuronal polarization in a fluctuating state. *Science Advances*, 8(44), eabo2336.

<https://doi.org/10.1126/sciadv.abo2336>

2.1. Summary

We used to think about the MT array in neurites as a large stationary structure that mainly acts as a railroad network that transports factors^{82,181,182} and is dynamic only on the level of single MTs, which can polymerize and depolymerize¹⁸³ as well as be generated^{37,84,85} and transported⁸². I challenged this view by showing that the entire MT array is instead very dynamic: it constantly moves from the neurite tip into the soma.

We expressed photoconverted tubulin in young developing primary mouse hippocampal neurons and then followed small photoconverted MT patches over time. To our surprise these MT patches were not stationary but constantly moved towards the soma at the considerable speed of 0.5 $\mu\text{m}/\text{min}$. At this early time in neuronal development, neurons are in a very dynamic and fluctuating state in which single neurites grow for a bit but then retract again^{11,13,166}. At some point a single neurite escapes this non-growing state by stochastically growing a lot, thereby becoming the axon. The other neurites are actively hindered from growing further by not well characterized mechanisms and will become dendrites. Since an axon must extend its MT array to grow¹⁸⁴, we asked whether MT-RF would slow down in the axon to enable axon growth. When following photoconverted MT patches in neurons with an axon we found that MT-RF is reduced by around two to three-fold. Importantly, we saw a similar slowdown in adherent cultures, neurons in 3D collagen matrices and neurons in brain slices.

2. Microtubule retrograde flow retains neuronal polarization in a fluctuating state

To understand what the role of the slowdown is, we next characterized at which time during the extended course of axon development it happens. To measure MT-RF long enough, we established imaging of the fluorescently labeled MT minus end-binding protein CAMSAP3. Slowdown specifically in the axon, compared to all other neurites happened in 67% of neurons. Importantly, slowdown happened around 30 minutes after initial axon outgrowth. This suggests that MT-RF slowdown could stabilize axon growth. Importantly, this could mean that the main role of MT-RF is to destabilize developing neurites after axon growth and all neurites before axon growth to keep them short.

A hallmark of the fluctuating state before axon development is that neurites when they grow also increase their MT density¹⁶⁸. This increased MT density goes back to baseline again when the neurite retracts. We measured MT density cycles before and after axon development and found that MT density cycles were reduced specifically in the axon. When looking at neurons before axon growth, we found that neurites with faster MT-RF also showed more MT density cycles. This suggests that MT density cycles are fueled by MT-RF, particularly before axon development and in developing neurites. We propose that MT-RF fuels MT density cycles by moving higher microtubule density out of neurites.

Since we propose that MT-RF stabilizes axon growth, we asked whether compounds that stabilize axon outgrowth in multiple neurites^{57,60} could act through slowing down MT-RF. For that purpose, we made use of two different compounds with completely different modes of action but which both stabilize axons. On the one hand side, we used para-amino blebbistatin which inhibits myosin-2 and on the other hand taxol which stabilizes MTs. We found that both compounds slowed down MT-RF to axonal levels and reduced MT density cycle. This is consistent with our hypothesis that MT-RF slowdown stabilizes axon identity through reducing MT density cycles.

We next wanted to look into the mechanism driving MT-RF. For this, we investigated whether the MT motor protein dynein could drive MT-RF. Inhibiting dynein both acutely with a small molecule and chronically by expressing a dominant negative domain, slowed down MT-RF. Additionally, recruiting endogenous dynein or overexpressed

dynein motor domain to the plasma membrane using chemical dimerization sped up MT-RF. If dynein fuels MT-RF from the plasma membrane, it would need to act on plus-end-in MTs, since it would move these retrogradely towards the soma. Therefore, we tested if recruiting dynein to the plasma membrane was also able to speed up MT-RF in axons, since axons have almost no plus-end-in MTs. In parallel, we checked whether this recruitment could speed up MT-RF in a dendrite of the same neuron, since half of all MTs are plus-end-in oriented in dendrites^{138,139}. While Dynein recruitment to the plasma membrane did not speed up MT-RF in axons, it did speed up MT-RF in dendrites. This indicates that Dynein at the plasma membrane speeds up MT-RF by acting on plus-end-in MTs.

Lastly, we tested the effect of faster MT-RF on neurons. For this purpose, we again recruited dynein to the plasma membrane and measured tubulin density and MT-RF simultaneously. In developing neurites, speeding up MT-RF by recruiting overexpressed dynein to the plasma membrane reduced MT density and induced neurite retraction. Interestingly, speeding up MT-RF in dendrites also massively reduced MT density. This shows that MT-RF reduces MT density and destabilizes neurite growth.

In summary, our discovery of MT-RF provides a completely novel level of cytoskeleton dynamics. This allows a new view on the physiology of neurons. Potentially, slow down of MT-RF could be impaired in neurodevelopmental disorders or MT-RF could be increased during neurodegenerative conditions, which provides new avenues to understand and treat pathology.

2.2. Statement of Contribution

Table 1: Statement of contribution for manuscript Schelski and Bradke, 2022.

Task	Author
Project idea	M.S.
Conceptualization	M.S., F.B.
Methodology	M.S.
Software	M.S.
Formal analysis	M.S.
Investigation	M.S.
Resources	M.S.
Writing - original draft	M.S.
Writing - review & editing	M.S., F.B.
Supervision, project administration and funding acquisition	F.B.

3. Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance

This chapter of the thesis represents a preprint that was posted on bioRxiv on 18th of December 2025¹⁸⁵. The manuscript is currently under review in the journal *Nature Communications*.

Schelski, M., Pietralla, T., Günter, C., Stern, S., Pavin, N., & Bradke, F. (2025). Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance. bioRxiv, 2025.12.18.694605.
<https://doi.org/10.64898/2025.12.18.694605>

3.1. Summary

Dendrites and axons were thought to develop through separate programs¹⁸⁶. We show here that one unifying mechanism directs the development of both axons and dendrites: slowdown of MT-RF. We develop a data-driven biophysical model of MT dynamics that incorporates polymerization, catastrophe, different mechanisms of nucleation, MT stabilization and MT-RF. Our model makes experimentally verified predictions about the MT array across developmental stages – from developing neurites to dendrites and axons. The model predicts that MT-RF mainly reduces stable MTs and distal MTs and that this effect is increased for higher stable MT half-lives. The increased reduction of MTs by MT-RF for longer lived stable MTs is intuitive since MT-RF moves these MTs further towards the soma during their longer lifetime and thereby reduces them more.

In developing neurites, we measured the distribution of unstable and stable MTs using quantitative immunocytochemistry. We stained for acetylated and detyrosinated tubulin as markers for older and thus stable MTs and for tyrosinated tubulin as a marker for younger and thus unstable MTs. We found that the distributions of detyrosinated and tyrosinated tubulin do not represent stable and unstable MTs well in developing neurites. Using the distribution of acetylated tubulin as a marker for stable MTs and calculating the distribution of non-acetylated tubulin as a marker for unstable MTs represented the distribution of stable and unstable MTs better. We also

measured the half-life and fraction of unstable and stable MTs by live-cell imaging of photoconverted MTs.

Our model can explain experimental MT decay and experimental distributions of unstable MT, stable MTs and growing MT tips in developing neurites. The model predicts that MT-RF mainly reduces stable distal MTs. To challenge our model, we then compared re-analyzed experimental raw data on MT loss after MT-RF speedup¹⁴² with model simulations without new fitting of parameter values and using the experimental timing and MT-RF speeds. Importantly, the model provides an explanation for the experimental MT reduction induced by speeding up MT-RF both globally and distally. This supports the larger effect of MT-RF on stable and on distal MTs.

In dendrites, we find experimentally that the half-life of stable MTs as well as the amount of stable MTs are increased compared to developing neurites. Our model predicts that this stable MT half-life increase allows a massive increase in stable MTs globally and distally, but only if MT-RF also slows down. The model predicts that without MT-RF slowdown, stable MTs are increased three-fold less by the higher stable MT half-life. Again, we test our model by comparing re-analyzed experimental raw data on MT loss after MT-RF speedup in dendrites¹⁴² with model predictions without additional parameter value fitting and using experimental timing and MT-RF speeds. The model predictions of global and distal MT loss match the experimental MT loss upon MT-RF speedup well. This supports the predicted effect of MT-RF on MTs in dendrites. MT-RF slowdown is needed for the high stable MT increase in dendrites through increased stable MT half-life.

In axons, the experimental distributions of unstable and stable MTs increase towards the axon tip. As expected, we find that the amount of stable MTs and the stable MT half-life increase while MT-RF slows down in axons. Additionally, it is known that MTs in the axon are uniformly plus-end-out oriented. One of the types of MT nucleation in the model, nucleation in the cytosol, is known to lead to a high degree of plus-end-in MTs and therefore is downregulated in axons¹⁵⁰. When the model incorporates all these changes, it can fit the distribution of stable MTs well, but only when also incorporating the known increase in MT branching in axons¹⁵⁰. The model

predicts that the increase in MT branching is needed for stable MTs to increase towards the axon tip. Importantly, without MT-RF slowdown, stable and unstable MTs always massively reduce towards the axon tip, even with high MT branching. Thus, the model predicts that MT-RF should slow down in order for higher MT branching to increase stable MTs towards the axon tip. Further, the model predicts that MT-RF slowdown is needed to maintain any MTs in the distal axon, with distal MTs being long-known key determinants of axon growth^{11,57,60,184}. This reveals a previously unknown mechanism that limits axonal length - through removing distal MTs without MT-RF slowdown.

Using our biophysical model, we next tested why the reduction of distal MTs by MT-RF is much stronger in the axon compared to developing neurites. We first checked the effect of the higher stable MT half-life in axons with fast MT-RF. Indeed, reverting stable MT half-life down to the value from developing neurites increases distal MTs in axons with faster MT-RF. We then tested the effect of the lower cytosolic nucleation rate in axons with fast MT-RF. With the higher cytosolic nucleation rate as in developing neurites, distal MTs are increased massively when MT-RF is fast. Thus, both the higher stable MT half-life but especially the lower cytosolic nucleation rate lead to a high reduction of distal MTs in axons. This suggests that axons need to slow down MT-RF to maintain distal MTs while also showing a large amount of stable MTs and uniform plus-end-out orientation.

In summary, MT-RF slowdown is a unifying mechanism that enables both axon and dendrite development and maintenance. Our data reveals a unifying model of how the intricate interplay of MT-RF with different molecular MT dynamics generates the polarized morphology of neurons across developmental stages.

3.2. Statement of Contribution

Table 2: Statement of contribution for manuscript Schelski et al., 2025.

Task	Author
Project idea	M.S.
Conceptualization	M.S., N.P.
Methodology	M.S., T.P., C.G., S.S., N.P.
Software	M.S.
Experiments	M.S., T.P., C.G., S.S.
Image analysis	M.S., C.G.
Experimental data analysis	M.S.
Model analysis	M.S.
Resources	M.S.
Writing - original draft	M.S.
Writing - review & editing	M.S., T.P., C.G., S.S., N.P., F.B.
Supervision, project administration and funding acquisition	N.P., F.B.

4. Summary

The MT array was thought to be the stationary backbone of neurons. Instead, we found that the MT array constantly moves from the tip into the soma retrogradely as a whole. This MT-RF enables neurons to maintain their highly dynamic fluctuating state before axon development, while its slowdown is needed for both axon and dendrite development. Thereby, MT-RF and its slowdown emerge as the center piece of a unifying model across developmental stages.

What keeps the MT array together to enable it flowing retrogradely as a whole? It was surprising to see all MTs moving retrogradely together. We found that when strongly depolymerizing actin with Latrunculin-A (LatA), MTs would not flow uniformly retrogradely but instead both anterogradely and retrogradely several fold faster than the speed of MT-RF, while a net retrograde bias for the movement remained. This suggests that actin keeps the MT array together, either by connecting MTs or by interacting with a complex that keeps MTs together. Adding up all the forces acting on MTs in the array in turn leads to a net retrograde force that drives the entire MT array retrogradely, MT-RF. Since actin was found to be present between MTs in neurons¹⁸⁷, actin might crosslink MTs to keep them together. This could also explain how bidirectional MT transport of single MTs is possible even with the entire MT array moving retrogradely: by loosening actin contacts of some neighboring MTs, these MTs can escape uniform retrograde movement. Thus, bidirectional MT transport and uniform MT-RF are not mutually exclusive.

Which mechanism fuels MT-RF? On the one hand side, I found that inhibiting myosin-II slowed down MT-RF. This could suggest that myosin-II contraction along the neurite or from the growth cone could push the MT array out of the neurite or that myosin-II contraction from the soma could pull MTs into the soma. Interestingly, recently myosin-II was found to be involved in moving MTs in mature axons of *C. elegans* as well, supporting a role of myosin-II in MT movement¹⁸⁸. In addition to myosin-II, our data suggests that dynein could physiologically contribute to MT-RF, as inhibiting dynein both acutely and chronically slowed down MT-RF. Since recruiting

4. Summary

dynein to the plasma membrane sped up MT-RF, endogenous dynein might fuel MT-RF from the plasma membrane. Interestingly, also recruiting exogenously expressed kinesins to the plasma membrane sped up MT-RF, albeit in a different fashion than dynein. While recruiting dynein led to a continuous speed up of MT-RF, recruiting kinesin, after an initial delay, moved all MTs very rapidly and suddenly to the soma. Still, since we don't have any data on inhibiting kinesin activity, it is not clear whether kinesins could play a role in fueling MT-RF physiologically. In summary, it is likely that physiologically both myosin-II and MT-based motor proteins, particularly dynein, contribute to MT-RF. However, further experiments are needed to delineate the physiological mechanisms that drive MT-RF. First of all, the physiological localization of dynein should be established to test whether it localizes to the plasma membrane in neurites, as we suggest. Further, knockout or knockdown of dynein, kinesins or myosin-II could reveal which MT-based motor is physiologically important for MT-RF.

How does MT-RF slow down in the axon? Three main mechanisms could play a role for MT-RF slowdown in the axon. First, since we found that myosin-II inhibition slows down MT-RF, reduced myosin-II activity in the axon⁵⁷ could slow down MT-RF. Second, since we found that dynein at the plasma membrane can act on minus-end-out MTs to move MTs retrogradely, uniform plus-end-out MT orientation in the axon could inhibit MT-RF. Third, since we found that taxol slows down MT-RF before it changes MT orientation, MT stabilization by itself could slow down MT-RF. Since MTs are stabilized in the axon⁶⁰, this might also lead to slowdown of MT-RF. However, each of these potential mechanisms requires further investigation to determine whether they play a role in MT-RF slowdown in axons.

By which molecular mechanism could MT-RF drive the fluctuating state while MT-RF slowdown in the axon subsequently reduces fluctuations? In neurons with developing neurites before axon development, stable MT half-life could stochastically increase. This increases the fraction of stable MTs and thereby recruits axon-specific factors, such as the MT motor protein kinesin-1, into the neurite through more detyrosinated MTs⁹⁴. This could lead to a positive feedback loop in which axon-specific factors could increase stable MT half-life further. However, with fast MT-RF, the

increase in stable MTs - driven by their extended half-life - is attenuated, breaking the feedback loop and therefore making the stable MT increase transient, as it is before axon and dendrite development⁹². In contrast, MT-RF slowdown in a young axon¹⁴² allows a steady increase in stable MTs through increased stable MT half-life, stabilizing axon identity. Recently, it was found that artificially slowing down MT-RF in all neurites before axon development directs kinesin-1 into all neurites and subsequently leads to axon development¹⁸⁹. This could be explained by MT-RF slowdown increasing stable MTs in neurites with increased MT half-life, which could direct kinesin-1 into all neurites due to increased detyrosination, thereby triggering axon development.

How could early MT-RF slowdown lead to axon development while late MT-RF slowdown leads to dendrite development? Since early in development stable MTs often are still attached to the centrosome, stable MTs are mainly plus-end-out, thereby inducing axon development¹⁹⁰. Since centrosomally nucleated MTs were found to be dispensable for axon growth¹⁹¹, other mechanisms could be more important in mediating MT nucleation from the soma for a preferred plus-end-out MT orientation. One alternative to the attachment to the centrosome could be MTs nucleating from the Golgi apparatus^{85,191,192}, which was shown to be important for axon length⁸⁵. We also know that additional mechanisms drive the uniform plus-end-out MT orientation specifically in axons: MTs are less likely to be nucleated in the cytosol¹⁵⁰ and dynein transports plus-end-in MTs out of the axon^{143,144,146,193}. When MT-RF later slows down in dendrites, MTs are detached from the centrosome⁸⁴ and, with time, become more plus-end-in oriented due to MT-based motor protein activity¹⁹⁰. It is possible that stable MTs are more likely to be plus-end-in oriented than unstable MTs since their longer half-life allows MT-based motors to act on them longer, making it more likely that MT-based motors change the orientation of stable MTs to plus-end-in. The resulting stable plus-end-in MTs lead to dendrite development. Thus, when MT-RF slowdown increases stable plus-end-out MTs, an axon is formed, while when it increases stable plus-end-in MTs, a dendrite forms.

4. Summary

My data highlights that the distribution of stable MTs changes drastically during the development of axons and dendrites compared to developing neurites and that these changes depend on the interplay of MT dynamics with MT-RF slowdown. Previously, it was shown that the distribution of the ratio of plus-end-out to plus-end-in MTs along dendrites influences transport dynamics¹⁴⁰. Since plus-end-out MTs are preferentially unstable and plus-end-in MTs preferentially stable¹⁴¹, a change in the ratio of stable to unstable MTs along the dendrite could also alter dendritic transport dynamics. Thereby, understanding the formation of stable MT distributions could help us decipher dendritic transport dynamics of proteins and mRNAs and their distributions, which is crucial for synaptic transmission and synaptic plasticity^{194–199}.

Our biophysical model allows not only to look at one mechanism in isolation but instead puts the mechanism into context with different MT dynamics and allows to investigate their interplay. This approach has already proven useful for the investigation of MT dynamics¹⁷⁵ and spindle MTs during cell division, with decades of research revealing the function of the spindle through the interplay of MT dynamics while also integrating the role of different motor proteins^{176,177,180,200}. Applying our biophysical model in neurons in different contexts could enable understanding the role of MTs depending on their localization and neuronal properties in physiology and pathology.

On the one hand, the effect of different proteins previously investigated, such as MT associated proteins (MAPs) in dendrites and axons (MAP2, MAP1B, Tau), could be pinned down and put into the context of their dendritic or axonal localization by measuring the effect of their depletion on MT dynamics. Implementing these measured MT dynamics into our biophysical model would allow to assess which measured change in MT dynamics contributes the most to the experimental change in stable and unstable MT density.

On the other hand, our biophysical model could also prove useful in understanding the role of MTs in neurodevelopmental and neurodegenerative diseases as well as neuroregeneration. For many neurodevelopmental^{201–203} and neurodegenerative diseases or spinal cord injury, MTs are affected, e.g. through a change in MT polarity²⁰⁴ or often through a reduction in MT mass in both dendrites and axons^{205–}

²⁰⁸. However, overstabilization of MTs, as in Hereditary Spastic Paraplegia, can also cause neurodegeneration²⁰⁷, suggesting that stabilizing MTs to increase MT mass might not always be the ideal treatment. As these conditions are complex phenotypes with many changes, it becomes difficult to pinpoint which pathological changes are mechanistically most detrimental. Measuring changes in MT dynamics systematically for pathological conditions and implementing these changes separately in our biophysical model would allow to pinpoint which changes have the most detrimental effect. This could benefit currently underway therapeutic studies of treating neurodegenerative diseases with compounds acting on MTs²⁰⁹ by suggesting context-specific treatments that target the most detrimental changes. Importantly, moderate MT stabilization is already known to enable axon regeneration, leading to functional recovery^{14,15,210,211}, and is suggested for the treatment of neurodevelopmental disorders²⁰², indicating a promising potential for exploiting the manipulation of MT dynamics for therapeutic interventions in neurological diseases. To identify the best combination of treatments, it could be promising to combine our data-driven biophysical model with novel cell-culture based screening systems of neurological diseases using human induced pluripotent stem cell (iPSC) derived neurons to test predictions of phenotypic changes for treatments in different disease conditions^{212–216}. Our data-driven biophysical model could help to quantitatively understand the mechanisms of MT array formation underlying MT defects in neurodevelopmental disorders, spinal cord injury and neurodegeneration and thereby open potential avenues of how to combat them most effectively.

The role we identified for MT-RF could extend beyond neurons. One interesting parallel for MT-RF to highlight is the similarities to MT flux during cell division. During cell division, MTs move towards the poles of the centrosomes at 0.6 $\mu\text{m}/\text{min}$ ²¹⁷. This poleward MT flux is crucial for chromosome positioning through regulating spindle length^{218,219}. This MT flux is remarkably similar to MT-RF in neurons which moves MTs retrogradely at 0.5 $\mu\text{m}/\text{min}$ and which we in this work found is important for the regulation of neurite length. These similarities suggest that retrogradely moving MTs is a conserved mechanism that enables length control in different biological contexts. Interestingly, during cell division the half-life of MTs changes in different phases and

can become as high as in neurons²²⁰. Our model suggests that MTs with a higher half-life would be more affected by MT flux. Additionally, MT flux also changes between mitotic phases^{220,221}. Our model suggests that these changes in MT flux could enable dynamic remodeling of the spindle, strongly mediated by MT half-life. In summary, transitions between phases of cell division could be regulated by MT half-life modulating the effect of MT flux on the spindle.

Considering the conserved role of retrograde MT flux to regulate length, even during cell division, could MT-RF also enable dynamic morphology changes in non-neuronal cell-types with MT-filled processes? Several cell types indeed have very dynamic MT-filled processes, like microglia^{222,223}, oligodendrocyte precursor cells (OPCs)^{224–226} and invading carcinoma cells²²⁷. In microglia, dynamic morphology changes of MT filled processes are important for cytokine-mediated inflammatory responses when microglia become reactive^{222,223}. OPCs need to change their morphology for myelination and interestingly MT stability is important for this process^{224,225}, which is similar to the role of MT stability for the growth of neurites. In carcinoma cells, MT-filled processes begin to form and become dynamic when carcinoma cells invade surrounding tissue while metastasizing²²⁷. Thus, the dynamics of MT-filled processes are crucial across many biological functions and during disease. Understanding whether MT-RF exists within these processes and whether MT-RF fuels their dynamics could allow entirely new approaches for fixing potential dysfunctions of microglia and OPCs and blocking metastasis during cancer.

Acronyms

3D	3-dimensional
ADP	Adenosine diphosphate
Akt	Protein kinase B
APC	Adenomatous polyposis coli
Arp2/3	Actin-related protein 2/3
ATP	Adenosine triphosphate
CAMSAP	Calmodulin-regulated spectrin-associated protein 3
Cdc42	Cell division control protein 42 homolog
C-domain	Central domain
C-KIFs	C-terminal motor domain KIFs
CLASP2	Cytoplasmic linker associated protein 2
CLASP	CLIP associating protein
CLIP	Cytoplasmic linker protein
CRMP2	Collapsing response mediator protein 2
DOCK7	Dedicator of Cytokinesis 7
Dync1h1	Dynein heavy chain family, cytoplasmic dynein heavy chain 1
Dync2h1	Cytoplasmic dynein heavy chain 2
EB1	End binding protein 1
EB3	End binding protein 3
F-Actin	Filamentous actin
G-actin	Globular actin
GSK3 β	Glycogen synthase kinase-3 β
γ -TuRC	Gamma-tubulin ring complex
GDP	Guanosine diphosphate
GEF	guanine nucleotide exchange factors
GTP	Guanosine triphosphate
HAUS	HAUS Augmin Like Complex Subunit 1
ILK	Interleukin-like kinase

iPSC	Induced pluripotent stem cell
KIF	Kinesin superfamily
L1-CAM	L1 Cell Adhesion Molecule
LKB1	Liver kinase B1
MAP	Microtubule associated protein
MAP1B	Microtubule associated protein 1b
MAP2	Microtubule associated protein 2
MARK	Microtubule-affinity regulating kinase 2
M-KIFs	Middle motor domain KIFs
MT	Microtubule
MT-RF	Microtubule retrograde flow
NGF	Nerve growth factor
N-KIFs	N-terminal motor domain KIFs
OPC	Oligodendrocyte precursor cell
PAK1	p21-activated kinase 1
P-domain	Peripheral domain
PCM	Pericentriolar material
PI3K	Phosphoinositide 3-kinase
Rac1	Ras-related C3 botulinum toxin substrate 1
Rho	Ras homologous
SAD	Synapses of amphids defective
T-domain	Transition domain
Tiam1	T-lymphoma invasion and metastasis-inducing protein 1
TRIM46	Tripartite motif containing 46
WASP1	Wiskott-Aldrich syndrome protein
WAVE	WASP-family vevprolin-homologous protein

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Appendix 1: Microtubule retrograde flow retains neuronal polarization in a fluctuating state

NEUROSCIENCE

Microtubule retrograde flow retains neuronal polarization in a fluctuating state

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In developing vertebrate neurons, a neurite is formed by more than a hundred microtubules. While individual microtubules are dynamic, the microtubule array has been regarded as stationary. Using live-cell imaging of neurons in culture or in brain slices, combined with photoconversion techniques and pharmacological manipulations, we uncovered that the microtubule array flows retrogradely within neurites to the soma. This flow drives cycles of microtubule density, a hallmark of the fluctuating state before axon formation, thereby inhibiting neurite growth. The motor protein dynein fuels this process. Shortly after axon formation, microtubule retrograde flow slows down in the axon, reducing microtubule density cycles and enabling axon extension. Thus, keeping neurites short is an active process. Microtubule retrograde flow is a previously unknown type of cytoskeletal dynamics, which changes the hitherto axon-centric view of neuronal polarization.

INTRODUCTION

Microtubules build the morphology of eukaryotic cells. They support the complex morphology of neurons by forming arrays, composed of more than 100 microtubules each (1). These parallel tracks transport cargo within neuronal projections, the neurites (2). Within the microtubule array, single microtubules are dynamic; they can be transported (3), generated (4, 5), as well as polymerize and depolymerize (6). The microtubular array in the neurite itself, however, has been regarded as a stationary structure, even during neuronal development (7, 8). We challenge this view by showing that the microtubule array in developing neurites flows retrogradely from the distal neurite tip to the cell body.

This discovery of microtubule retrograde flow (MT-RF) changes our view on cytoskeletal dynamics in neurons. We demonstrate that this novel view transforms our understanding of neuronal polarization—the process of how neurons single out one neurite to become the axon, whereas the other neurites later become dendrites (9, 10). Formerly, it was thought that enrichment of specific signaling components distinguishes a rapidly growing axon from stationary neurites (10–12). Our data instead reveal that keeping neurites short is an active process, executed by MT-RF. In the axon, MT-RF is slowed down, enabling robust extension of the microtubule array and, thereby, neuronal polarization. Thus, the newly discovered type of cytoskeletal dynamics controls basic neuronal function. MT-RF might not only be specific to neurons but also influence microtubule-filled processes of non-neuronal cells.

RESULTS

The microtubule array in neurites flows retrogradely

To visualize the dynamics of the microtubule array in neurites of developing murine hippocampal neurons, we expressed a microtubule subunit (tubulin) fused to the fluorophore mEos3.2 (13), which can be photoconverted with ultraviolet light from green to red fluorescence.

Through laser-induced local photoconversion of the fluorophore in neurites, we labeled small microtubule patches throughout the whole microtubule stack (fig. S1, A and B) and followed the patches (Fig. 1A) before axon formation. In contrast to what is expected from a stationary microtubule array, the patches quickly moved retrogradely within neurites toward the soma, at speeds of $0.53 \pm 0.03 \mu\text{m}/\text{min}$ (mean $\pm$ SEM; Fig. 1, B and C, and movie S1). Immobile and anterogradely moving patches were only observed in 4.7 and 1.8% of neurites, respectively (fig. S2, A and B). The speed of MT-RF is slower than microtubule polymerization (12 to 18 $\mu\text{m}/\text{min}$) (14, 15) and fast axonal transport (70 to 280 $\mu\text{m}/\text{min}$) (16) but in the range of slow axonal transport (0.2 to 5.5 $\mu\text{m}/\text{min}$) (8). MT-RF also occurred in the absence of neurite retraction (fig. S2C). To test whether the entire microtubule array in the neurite moved retrogradely, we followed multiple microtubule patches in the same neurite. To avoid a light-induced stress reaction in the neurite, tubulin was fused to the photoactivatable fluorophore Dronpa (17), which needs less light intensity than mEos3.2 to be photoactivated (Fig. 1D). The photoactivated microtubule patches moved retrogradely in a synchronous manner, maintaining a similar distance between each other (Fig. 1, E and F, and movie S2). Accordingly, the speed of retrograde flow of distal patches was, on average, only 12.5% faster than proximal patches (fig. S3, A and B). The actin cytoskeleton appears to be involved in keeping microtubules connected in an array. Photoactivated microtubule patches dispersed both retrogradely and anterogradely more quickly when cells were treated with the actin-depolymerizing drug latrunculin A (LatA; fig. S4, A to D) (18). Together, before axon formation, the microtubule array in a neurite flows retrogradely toward the cell body.

The retrograde flow of the microtubular array is slow in the axon

Microtubules polymerize into the neurite tip to extend the neurite (19), fueled by tubulin that both diffuses (20) and is transported (21) from the cell body. As neurites remain short before neuronal polarization and after axon formation (10, 22), it raised the possibility that MT-RF might restrain neurite growth by reducing the net extension of the microtubule array. Indeed, MT-RF could be an important hurdle for neurite extension, because MT-RF is 6 to 15 times faster than neurite or axon growth (5, 9, 23). Consequently, during axon

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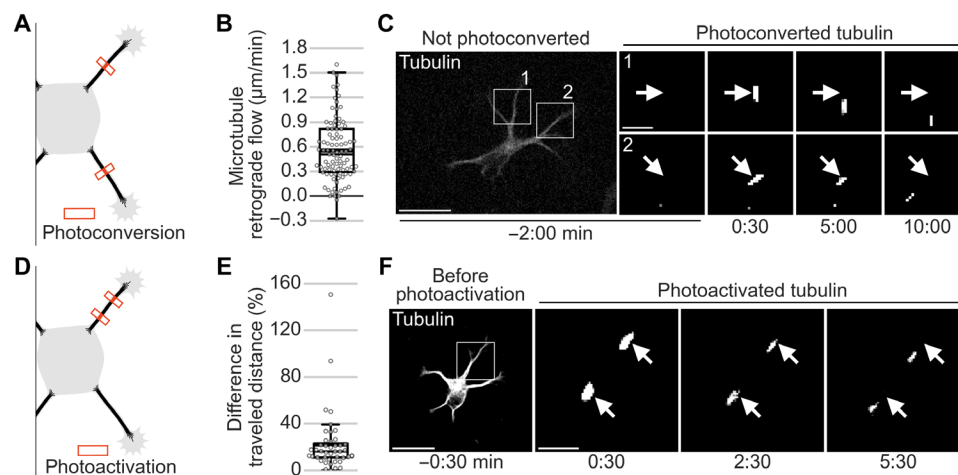


Fig. 1. The entire microtubule array in the neurite flows retrogradely before axon growth. The tubulin subtype TUBB2a was fused either (A to C) to the photoconvertible fluorophore mEos3.2 or (D to F) to the photoactivatable fluorophore Dronpa expressed in neurons and imaged after 1 day in culture. (A) Illustration of photoconversion experiment for (B) and (C). (B and C) Small microtubule patches were photoconverted in two neurites of neurons without an axon. (B) MT-RF in neurons without axons ($n = 71$ cells, $N = 11$ independent experiments). Each data point represents one neurite. (C) Representative cell for (B). (D) Illustration of photoactivation experiment for (E) and (F). (E) Two or three microtubule patches were photoactivated in a single neurite. For each neurite, the distance difference of the patch that moved furthest and the patch that moved the least was divided by the lower distance ($n = 44$ cells, $N = 9$ independent experiments). (F) Representative cell for (E). The thick line in boxplots shows mean. Scale bars, 20 μm (overview images) and 5 μm (zoomed images).

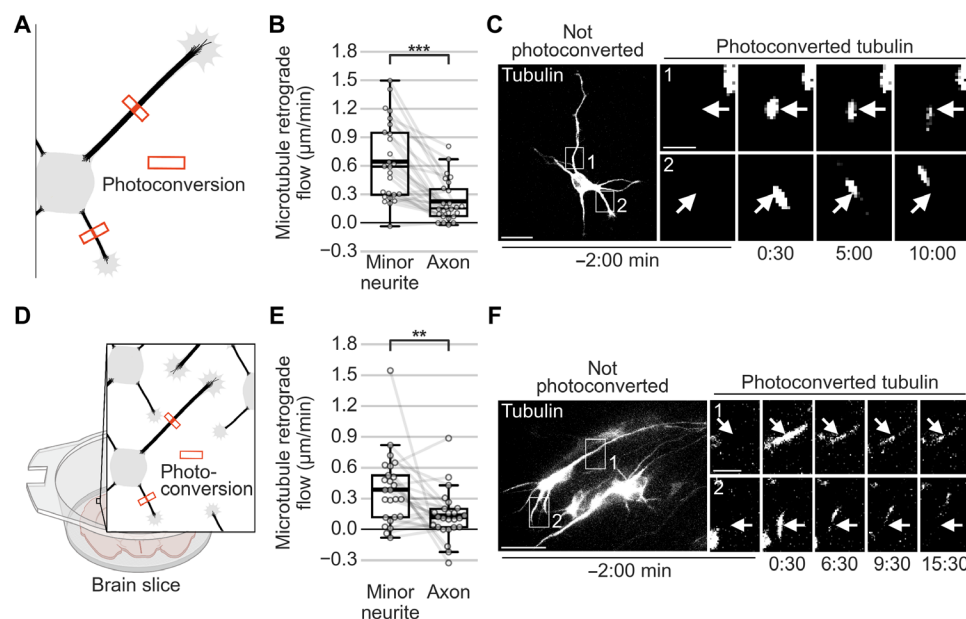


Fig. 2. MT-RF slows down in the axon. The tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2 expressed in neurons and imaged after 1 day in culture. (A) Illustration of photoconversion experiment for (B) and (C). (B and C) MT-RF in minor neurites and axons ($n = 26$ cells, $N = 8$ independent experiments). Arrows indicate location of photoconversion at 0:00 min. (D to F) Embryonic brains were electroporated ex utero at embryonic day 14.5 (E14.5) with tubulin subtype TUBB2a, fused to three times photoconvertible fluorophore mEos3.2. Brains were then sliced coronally, kept in the incubator for 2 days, and then imaged. (D) Illustration of the photoconversion experiment for (E) and (F). (E) MT-RF of neurons in brain slices. ($n = 25$ cells, $N = 8$ independent experiments). (F) Maximum intensity projection of the neuron photoconverted at 0:00 min, with arrows pointing to the photoconversion site. The thick line in boxplots shows mean. Arrows indicate the location of photoconversion at 0:00 min. $**P < 0.01$ and $***P < 0.001$, Wilcoxon signed-rank test. Scale bars, 20 μm (overview images) and 5 μm (zoomed images).

outgrowth, MT-RF may slow down in the axon to enable accelerated growth. Photoconversion of microtubule patches in minor neurites of axon-bearing neurons revealed that MT-RF was similar compared to the neurites of neurons without an axon. However, in the axon, MT-RF was more than twofold slower ($0.61 \pm 0.08 \mu\text{m}/\text{min}$ in minor

neurites compared to $0.23 \pm 0.05 \mu\text{m}/\text{min}$ in the axon; mean $\pm$ SEM; Fig. 2, A to C, and movie S3). The flow was similar across the entire axon length, as two photoconverted microtubule patches far away from each other moved at similar speeds (fig. S5, A to C, and movie S4). In addition, axons of neurons cultured in three dimensions (3D)

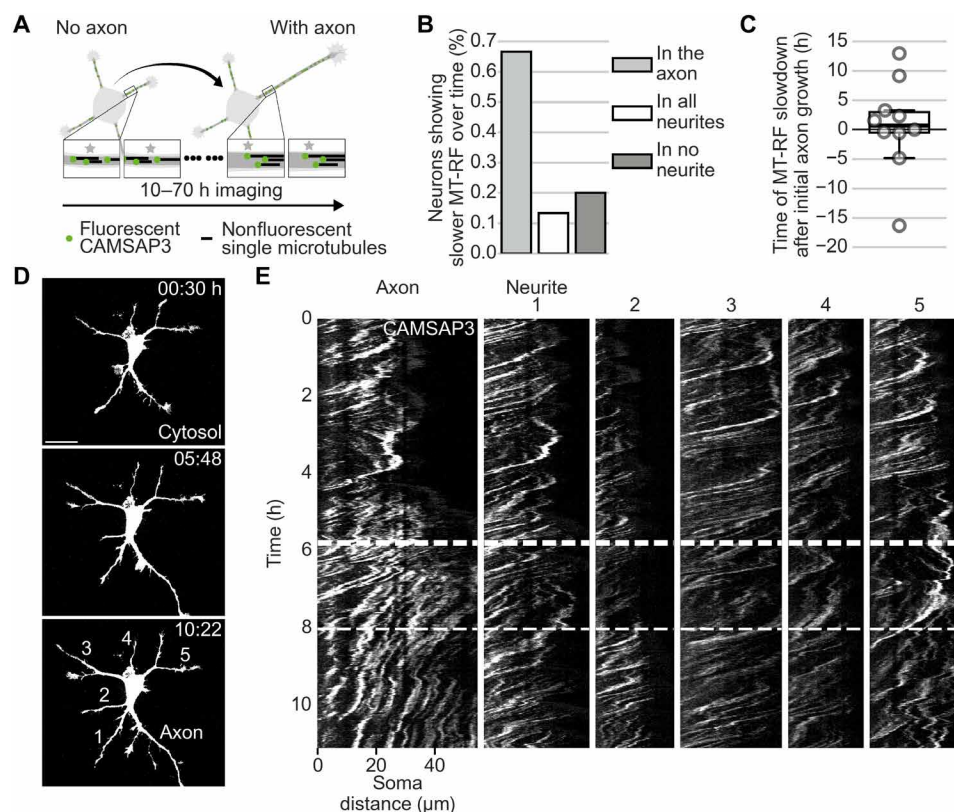


Fig. 3. MT-RF slows down shortly after axon growth. CAMSAP3 was fused to the fluorophore mNeonGreen and the cytosolic fluorophore tandem-mCherry (cytosol) expressed in neurons and imaged after 1 day in culture. **(A)** Illustration of long-term imaging of MT-RF by imaging the microtubule (minus) end-binding protein CAMSAP3 for **(B)** and **(C)**. **(B and C)** MT-RF was averaged over 200 min and was considered slowed down once it was 20% slower in the axon compared to all other neurites for 95% of time points for at least 180 min ($n = 15$ cells, from those, 10 cells for time of MT-RF slowdown, $N = 6$ independent experiments). **(D)** Cell morphology of neuron from **(E)** with the fluorophore tandem-mCherry to label the cytosol before (0:30 hours) and after (5:48 hours) the axon reached axon-like length and when MT-RF slowed down in the axon (10:22 hours). Neurites from **(E)** are labeled at 10:22 hours. **(E)** Neon-CAMSAP3 intensity along each neurite (x axis) over time (y axis; kymograph). The left site is close to the soma. Thus, a trace from top right to lower left indicates MT-RF. The thick and thin dashed lines indicate the time when the future axon reached axon-like length (5:48 hours) and when MT-RF slowed down in the axon compared to all other neurites (8:02 hours), respectively. The thick line in boxplots shows mean. Scale bar, 20 μm .

(24) and of neurons in embryonic organotypic brain slices showed slower MT-RF than minor neurites (Fig. 2, D to F; fig. S6, A to C; and movies S5 and S6). Notably, at later developmental stages, when neurites grow as dendrites (9), dendritic MT-RF speed decreased, but not to the level of that found in the axon (fig. S7, A and B, and movie S7).

We then defined when MT-RF slowed down in relation to axon formation. As repeated photoconversions are toxic to neurons, we measured MT-RF during neuronal polarization in all neurites simultaneously by expressing and imaging the microtubule minus-end marker calmodulin-regulated spectrin-associated protein 3 (CAMSAP3) (25) fused to a fluorophore (Fig. 3A) (14). MT-RF slowed down in the axon compared to all other neurites in 66.7% of neurons (Fig. 3B). In these neurons, MT-RF decreased relative to all other neurites approximately 40 min after the initial axon growth (Fig. 3, C to E, and movie S8). Thus, the slowdown of MT-RF in the growing axon could enable its steady growth.

The retrograde flow of the microtubule array keeps neurons in a fluctuating state

Before axon formation, neurites fluctuate by transiently acquiring axon-like properties (Fig. 4A) (10, 26, 27). MT-RF could be the basis

of the fluctuating state by continuously acting on all neurites and thereby resetting the repeatedly acquired axon-like state, making this state transient. To label axon-like neurites, we used the constitutively active motor domain of kinesin 1 family proteins (caKIF5C), a key marker for axon-like properties that labels neurite tips and, after axon formation, persistently labels the tip of the axon (Fig. 4A) (26). Before axon formation, MT-RF was indistinguishable in neurites that accumulate caKIF5C compared to neurites that did not accumulate this marker (Fig. 4, B to D, and movie S9). In contrast, MT-RF decreased at the transition of axon formation in neurites with caKIF5C accumulation (fig. S8, A to C). Thus, persistent MT-RF slowdown is specific to the axon and does not occur in neurites with axon-like properties. This suggests that before axon formation, MT-RF continuously acts on neurites and could keep neurons in an unpolarized, fluctuating state.

During fluctuations, microtubules cycle between low and high density in different neurites (Fig. 5A). While higher density is caused by transient increases in microtubule polymerization (27), using the supply of tubulin diffusing and transported from the cell body, the mechanism underlying the decrease of microtubule density has remained elusive. We hypothesized that MT-RF could continuously

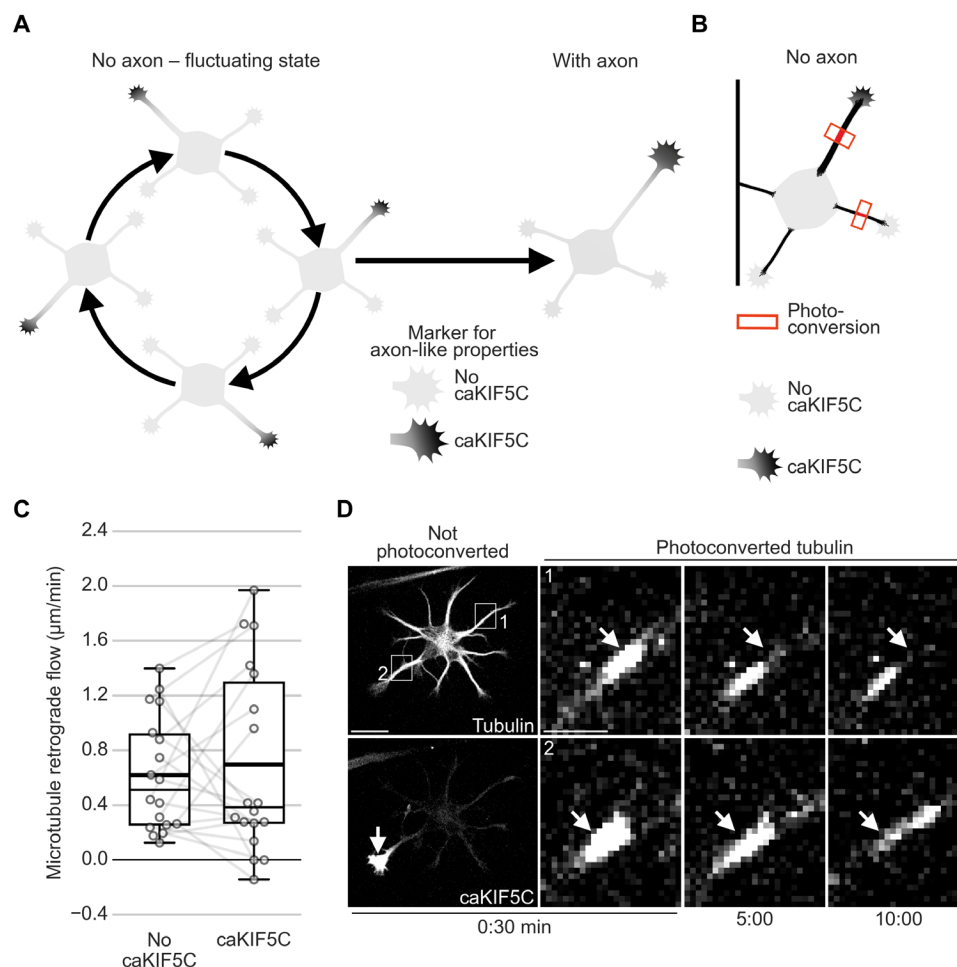


Fig. 4. MT-RF does not slow down in neurites with axon-like properties. Neurons expressing the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2, and caKIF5C fused to the fluorophore Cerulean3, were cultured for 1 day and then imaged. **(A)** Illustration of fluctuations of caKIF5C before axon formation. **(B)** Illustration of photoconversion experiments for **(C)** and **(D)**. **(C and D)** MT-RF quantified in neurites without and with caKIF5C accumulation in neurons without axons ($n = 18$ cells, $N = 8$ independent experiments). White arrows in the photoconverted channel of **(D)** indicate areas of photoconversion and, in the caKIF5C channel, point to the growth cone with caKIF5C accumulation. The thick line in boxplots shows mean. $P > 0.05$, Wilcoxon signed-rank test. Scale bars, 20 μm (overview images) and 5 μm (zoomed images).

move microtubules out of the neurite, reducing repeatedly increased microtubule density and thereby fueling microtubule density cycles. Consistent with this hypothesis, axons showed two times fewer microtubule density cycles than minor neurites (Fig. 5, B to D; fig. S9, A to C; and movie S10). In addition, we found an increase in microtubule density cycles in neurites with faster MT-RF before axon formation (Pearson $r = 0.46$; Fig. 5E and fig. S10, A to E).

This raised the possibility that fast MT-RF could prevent neurites from becoming axons by continuously counteracting microtubule accumulation. Consistent with this view, two pharmacological manipulations that transform nongrowing neurites into multiple growing axons per cell (23, 28) slowed down MT-RF in neurites. Even before axon growth, both para-amino blebbistatin (pa-Blebb) and taxol decreased MT-RF to the level of control-treated axons (Fig. 6, A to C, and movie S11). We therefore tested whether these manipulations, in addition to slowing down MT-RF, also decreased microtubule density cycles. Indeed, pa-Blebb and taxol reduced the number of

microtubule density cycles by approximately 50% (0.56 ± 0.03 cycles/hour for control, 0.24 ± 0.02 cycles/hour for pa-Blebb, and 0.29 ± 0.02 cycles/hour for taxol; mean $\pm$ SEM; Fig. 7, A to D, and movie S12). Thus, slower MT-RF could reduce microtubule density cycles and enable neurites to become axons by hindering the retrograde removal of microtubules from neurites.

Dynein recruitment to the plasma membrane causes speedup of MT-RF

Last, we investigated the involvement of microtubule-based motor proteins in driving MT-RF. As neurites contain microtubules in both orientations, 30% facing with their plus end toward the soma (plus-end-in) and 70% facing with their plus ends toward the tip (plus-end-out) (29), both minus end- and plus end-directed motors could be involved. Inhibiting the minus end-directed motor protein dynein slowed down MT-RF, both acutely following treatment with the small-molecule ciliobrevin A (30) and chronically following overexpression of the dominant negative-acting N-terminal domain of

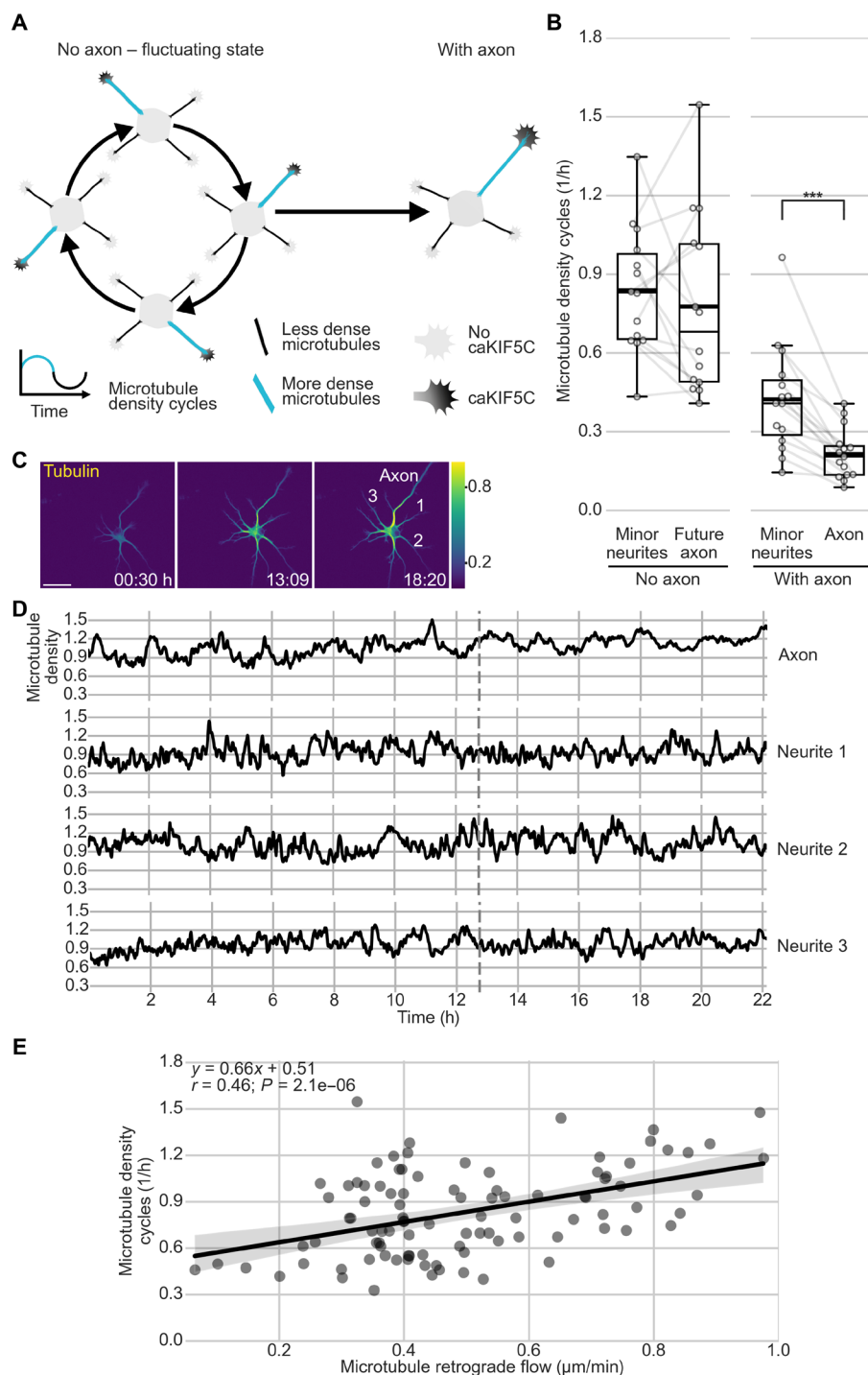


Fig. 5. MT-RF could keep neurons in the fluctuating state. Neurons expressing the fluorophore mNeonGreen fused to CAMSAP3 and TUBB2a fused to the fluorophore mScarlet were cultured for 1 day. Then, microtubules and MT-RF were imaged simultaneously in neurons from before to after axon formation. Microtubule density was calculated by dividing the average tubulin intensity by the tubulin expression level in the neuron, modeled as a quadratic function of the average intensity in all neurites for each time point. Changes of average microtubule density in a neurite of 0.2 or more were considered half a cycle. For a full cycle, an increase followed by a decrease, or vice versa, was required [$n = 15$ cells after axon growth (With axon), of those $n = 14$ cells before axon growth (No axon), $N = 3$ independent experiments]. **(A)** Illustration of fluctuations of microtubule density and caKIF5C before axon formation. **(B)** Microtubule density cycles in neurons before and after axon formation. Microtubule density cycles were averaged for all minor neurites of one neuron. **(C and D)** Representative cell for **(B)**. **(C)** Morphology and color-coded tubulin intensity (blue, lower intensity; yellow, higher intensity) at the start of imaging (00:30 hours), directly after (13:09 hours), and some hours after (18:20 hours) axon growth. Neurite numbers from **(D)** are annotated at the last time frame. **(D)** Microtubule density was smoothed with a rolling window of 2. The dashed line indicates the time of axon formation. **(E)** Correlation and linear regression of MT-RF with microtubule density cycles (Pearson $r = 0.46$, $P = 0.0000021$; $n = 95$ neurites from 14 cells, $N = 3$ independent experiments). The thick line in boxplots shows mean. *** $P < 0.001$, Wilcoxon signed-rank test. Scale bar, 20 μm .

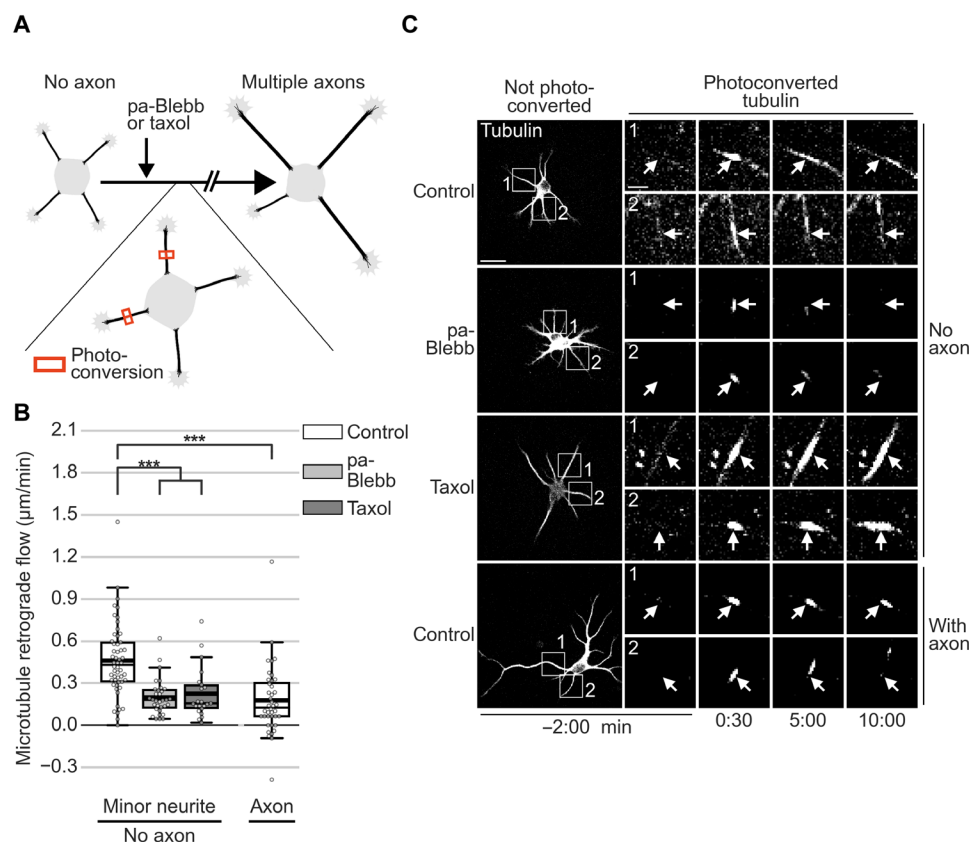


Fig. 6. Stabilizing axon identity in multiple neurites slows down MT-RF. The tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2 was expressed in neurons and imaged after 1 day in culture directly after treating neurons for 20 to 240 min with pa-Blebb (40 μM), taxol (6 nM), or control [dimethyl sulfoxide (DMSO)]. **(A)** Illustration of photoconversion experiment after drug treatments shown in **(B)** and **(C)**. **(B and C)** MT-RF after pa-Blebb, taxol, and control treatment ($n = 22$ cells, $N = 4$ for taxol; $n = 32$ cells, $N = 4$ for pa-Blebb; $n = 54$ cells, $N = 11$ for control without axon; and $n = 40$ cells, $N = 9$ for control with axon; N , number of independent experiments). **(C)** Arrows indicate location of photoconversion at 0:00 min. *** $P < 0.001$, Kruskal-Wallis multiple comparison with Dunn's post hoc test with Bonferroni correction. Scale bars, 20 μm (overview images) and 5 μm (zoomed images).

the dynein intermediate chain 2 (IC2N; Fig. 8, A to E, and movie S13) (31).

Dynein has diverse functions, including transporting cargo on microtubules (32); its global disruption induces neurite retraction through myosin II activation (33). Hence, global manipulation of dynein is not suitable to test the influence of MT-RF on neurons. We hypothesized that membrane-bound dynein might move microtubules and could thereby directly drive MT-RF. To test this, we used a well-established pharmacogenetic system (34) to recruit dynein to the plasma membrane upon addition of a dimerizer (Fig. 9A) and measured MT-RF. We started by recruiting endogenous dynein to the plasma membrane. We did this by overexpressing the N-terminal domain of the dynein adaptor bicaudal-D2 (bicDN), which binds dynein but cannot bind cargo (35), and then recruited bicDN itself to the plasma membrane (Fig. 9B). As bicDN inhibits dynein (36), expression of bicDN slowed down MT-RF. However, upon induced recruitment to the plasma membrane, MT-RF markedly increased (Fig. 9, C and D, and movie S14). We then expressed and recruited the dynein motor domain to the plasma membrane. When photoconverting microtubule patches, this recruitment moved $84 \pm 5\%$ of the photoconverted microtubule mass retrogradely, compared to $10 \pm 4\%$ anterogradely and $6 \pm 2\%$ remaining stationary (mean $\pm$ SEM; fig. S11, A and B). However, 20 to 30 min after dynein motor membrane

recruitment, this speedup became less uniformly retrograde (fig. S11, C and D). Hence, dynein activity at the plasma membrane is sufficient to transiently fuel uniform MT-RF.

As a minus end-directed motor, dynein at the membrane could exert a retrogradely directed force on plus-end-in microtubules. Consistently, in the axon of neurons cultured for 7 days, which contains less than 5% plus-end-in microtubules (29), dynein motor membrane recruitment did not increase MT-RF (fig. S12, A to D, and movie S15). Conversely, in dendrites, which contain 50% plus-end-in microtubules (29), dynein motor recruitment sped up MT-RF and reduced microtubule density (fig. S12, A to D, and movie S15). This suggests that the speedup of MT-RF by dynein at the membrane depends on the presence of plus-end-in microtubules.

The speedup of MT-RF by membrane-recruited dynein was linked to the motor function of dynein. Expressing and recruiting the wild-type dynein motor domain to the membrane sped up MT-RF, while MT-RF remained unchanged upon expression and recruitment of an inactive dynein motor domain (Fig. 9, E to G, and movie S16) (37). We also observed MT-RF acceleration with another minus end-directed motor, KIFC1, upon recruitment to the plasma membrane (fig. S13, A to C, and movie S17). The action of KIFC1 on MT-RF also depended on a functional motor domain, as a motor-deficient mutant (38) did not show such a speedup.

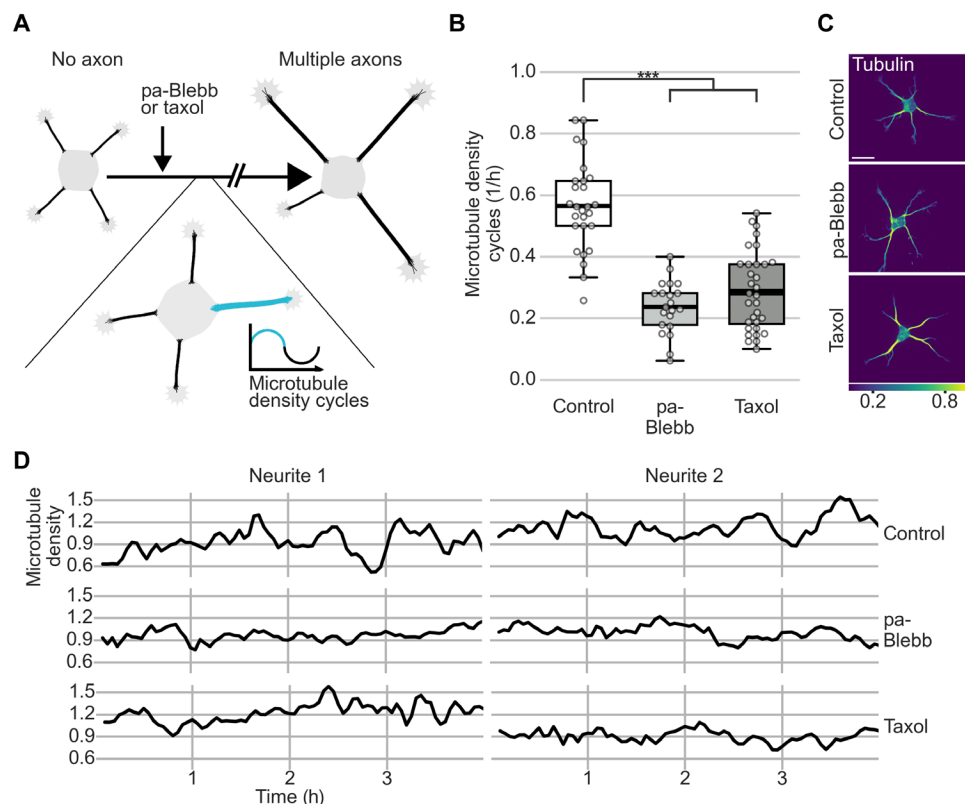


Fig. 7. Stabilizing axon identity in multiple neurites reduces microtubule density cycles. The tubulin subtype TUBB2a fused to the fluorophore mNeonGreen was expressed in neurons and imaged after 1 day in culture directly after treating neurons for 20 to 240 min with pa-Blebb (40 μ M), taxol (6 nM), or control (DMSO). (A) Illustration of experiment after drug treatments shown in (B) and (C). (B to D) Microtubule density was obtained by normalizing the average tubulin intensity by the tubulin expression level in the neuron, modeled as a linear function of the average intensity in all neurites for each time point. Microtubule density cycles were measured by counting the density increases followed by decreases in neurites of at least 0.2. Neurons were traced, and average intensity along neurites was measured using a self-made fully automated algorithm. (B) The number of cycles per hour was averaged for all neurites of a neuron before axon formation ($n = 34$ cells for taxol, $N = 3$; $n = 21$ cells for pa-Blebb, $N = 2$; and $n = 27$ cells for control, $N = 3$). (C) Morphology and color-coded tubulin intensity (blue, lower intensity; yellow, higher intensity) of neurons at the start of imaging. (D) Microtubule density was smoothed with a rolling window of two frames. The thick line in boxplots shows mean. *** $P < 0.001$, Kruskal-Wallis multiple comparison with Dunn's post hoc test with Bonferroni correction. Scale bar, 20 μ m.

Importantly, neurites with a speedup of MT-RF after recruiting the dynein motor domain to the plasma membrane (Fig. 10, A and B) showed enhanced immediate loss of microtubule density, similar to the effect found in dendrites (fig. S12C), and neurite retraction (Fig. 10, C to F, and movie S18). Thus, MT-RF keeps minor neurites short by reducing microtubule density and can thereby fuel microtubule density cycles (Fig. 10G).

The actin cytoskeleton plays a role in the speedup through the plasma membrane–recruited dynein motor domain. After actin depolymerization, recruited dynein motors moved microtubules more anterogradely, albeit a large fraction still moved retrogradely (fig. S14, A to D, and movies S19 and S20). The increase in anterograde movement was marked by a drastic increase of tubulin concentration in the distal neurite (fig. S14E and movie S20). Concomitantly, neurites rapidly extended for the next hour (fig. S14, F to H, and movie S20). This effect appears not to be caused by a change in microtubule orientation. Tracing the microtubule polymerization direction through fluorescently labeled microtubule plus-end binding 3 (EB3) protein revealed that actin depolymerization only changed microtubule orientation by 2% (fig. S15, A and B, and movie S21). In addition, pa-Blebb and taxol, both of which slowed down MT-RF,

did not change microtubule orientation (fig. S15, A and B, and movie S21).

Similar to dynein and KIFC1, expression and recruitment of the plus end–directed motor protein domains of KIF1a and KIF13a to the plasma membrane also sped up MT-RF. However, upon recruitment, both kinesin motor domain constructs first moved into the soma and then briefly sped up MT-RF while also retracting neurites (fig. S16, A to I, and movie S22).

In summary, our data reveal that the microtubule array of neurites moves retrogradely toward the soma in young developing neurons. MT-RF fuels microtubule density fluctuations in neurites by reducing repeated increases in microtubule density. MT-RF subsequently slows down in the axon and thereby stabilizes microtubule density fluctuations, allowing stable axon extension. Our experiments pinpoint motor proteins as potential molecular regulators of MT-RF (Fig. 10G).

DISCUSSION

Our work uncovers a previously unknown type of cytoskeletal dynamics, MT-RF. Through this mechanism, the microtubule array in

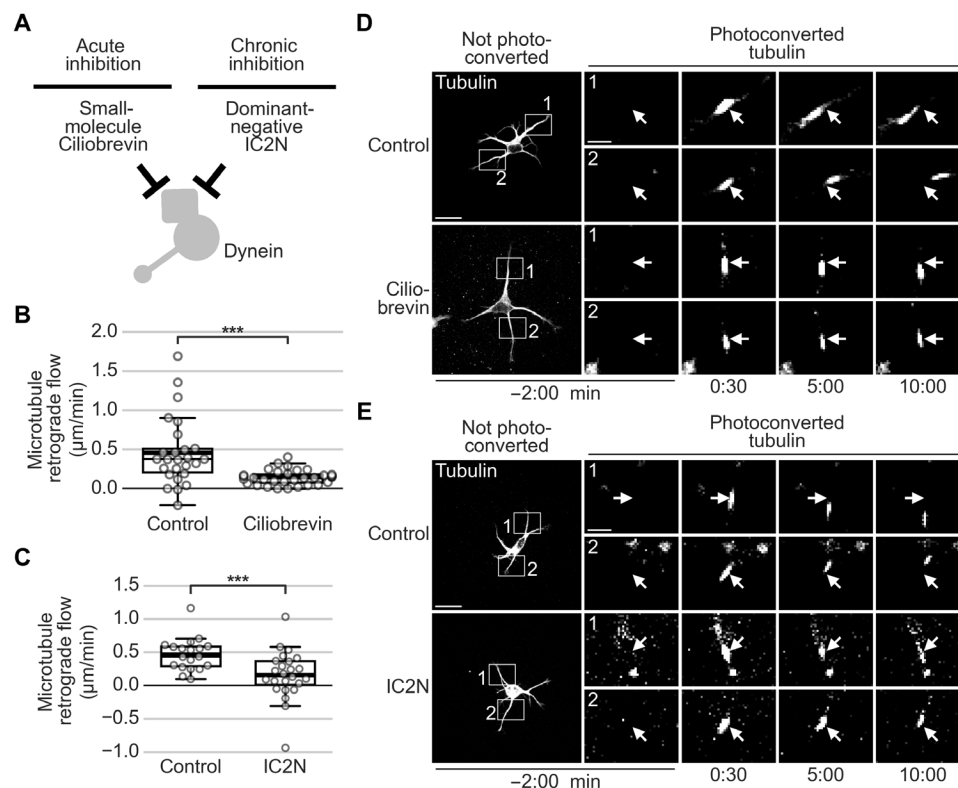


Fig. 8. Dynein inhibition slows down MT-RF. Neurons expressed the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2 and were imaged after 1 day in culture. **(A)** Illustration of experiments. **(B)** Neurons were treated with 50 μM ciliobrevin A for 30 to 60 min and then imaged ($n = 26$ cells, $N = 3$ for control; $n = 32$ cells, $N = 4$ for ciliobrevin). **(C)** Neurons expressed the Cerulean3-labeled N-terminal 234 amino acids of the dynein intermediate chain 2 (IC2N), which inhibits dynein through the dynein-dynactin interaction ($n = 19$ cells, $N = 3$ for control; $n = 24$ cells, $N = 4$ for IC2N). **(D and E)** Representative neurons for **(D)** ciliobrevin treatment in **(B)** and for **(E)** IC2N expression in **(C)**. Arrows indicate the location of photoconversion at 0:00 min. *** $P < 0.001$, Dunn's test. Scale bars, 20 μm (overview images) and 5 μm (zoomed images).

neurites is not stationary, but instead constantly moves toward the soma during early development. Tubulin diffuses (20) or is transported (21) from the cell body to integrate into polymerizing microtubules in neurites. The whole microtubule array then retrogradely moves toward the cell body. While its mechanisms remain to be investigated, a working model of MT-RF emerges, which we describe in the following sections. We will pinpoint the questions that arise from this discovery and the implications this has for our understanding of neuronal polarization.

Working model of MT-RF

As MT-RF is enhanced by minus end-directed membrane-bound dynein and KIFC1 motors, plus-end-in microtubules seem to play a prominent role in this process. But how can a mixed microtubule array present in neurites (29) enable uniform retrograde flow of the microtubule array? First, bundled and cross-linked microtubules in neurites (2, 39) could transmit the force exerted on a subset of microtubules to the entire microtubule array. The bidirectional microtubule flow we observed after actin depolymerization suggests that actin can keep microtubules together for uniform retrograde flow, potentially through actin-microtubule cross-linking proteins (7). Such a mechanism remains to be investigated in future studies.

Then, for a net retrogradely directed force, one possibility could be that plus-end-in microtubules are not equally distributed throughout the neurite diameter, but rather concentrate along sites close to

the plasma membrane. This could result in most plus-end-in microtubules close to the plasma membrane. Cryo-preserved electron microscopy and novel super-resolution fluorescence microscopy techniques (40, 41) will help to characterize microtubule distribution and geometry within neurites.

Alternatively, posttranslational modifications more common on plus-end-in microtubules might make them a better substrate for minus end-directed motors. However, this preference would need to be similar for at least the two minus-end motor domains tested in this study, dynein and KIFC1, which may render this possibility less likely. Future investigations need to pinpoint the physiological location and action of the motor proteins involved in MT-RF.

We showed that pa-Blebb and taxol cause MT-RF slowdown, yet neither treatment affected microtubule orientation during this time frame, suggesting a different mechanism for this process. Taxol lowers the critical concentration for microtubule assembly (42) and increases the rescue rates of microtubule instability (43, 44). By stabilizing microtubules, taxol also changes posttranslational modifications (45). Pa-Blebb is a myosin II inhibitor and decreases actin contractility (46–48) in axons and in actin arcs in the growth cone (23, 49, 50), thereby enabling microtubules to enter neurite tips more distally. Pa-Blebb also reduces neurite retraction (51). Myosin II might fuel MT-RF by increasing contractility in the neurite shaft or growth cone. Note that the mechanism governing how retrogradely moving microtubules are disassembled at the cell body remains to be studied. When

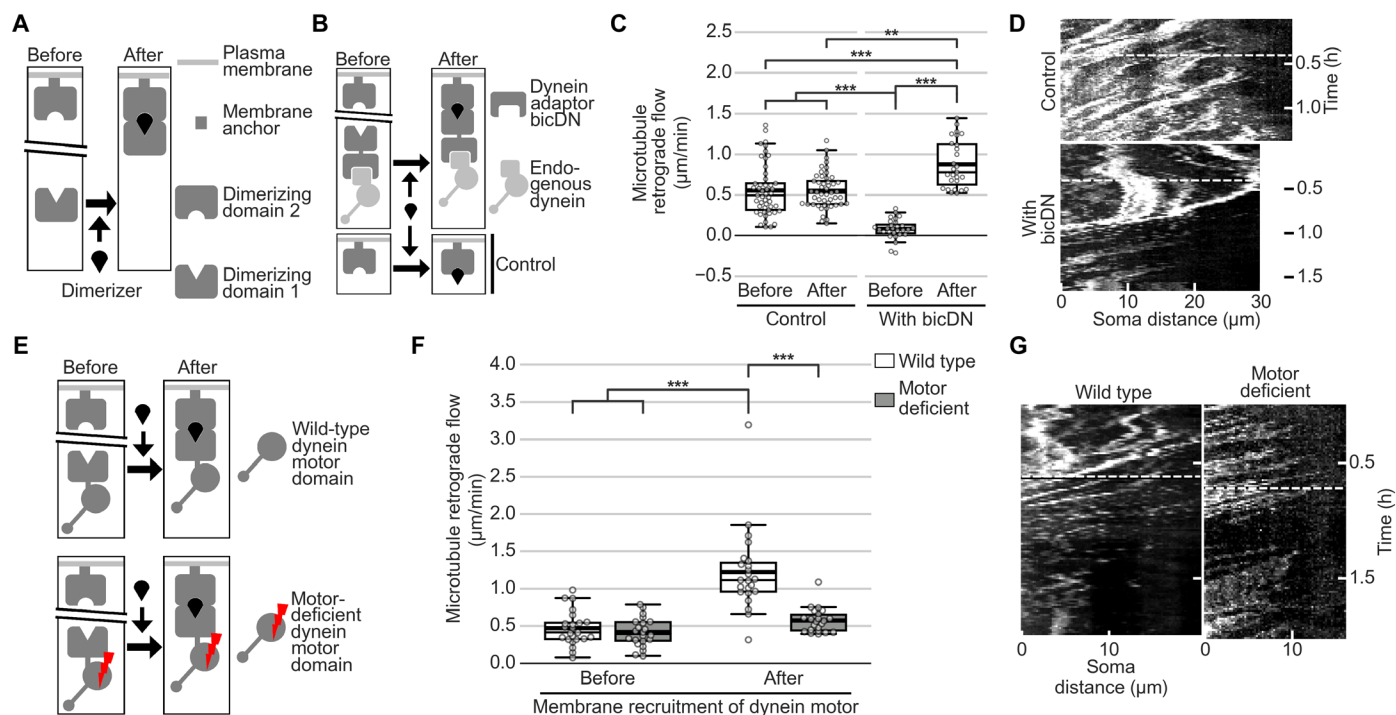


Fig. 9. Dynein at the plasma membrane speeds up MT-RF. Neurons expressed the fluorophore mNeonGreen fused to CAMSAP3 and were imaged after 1 day in culture. (A) Illustration of the chemical dimerization used to recruit proteins to the plasma membrane. Dimerization domains 1 and 2 are FK506-binding protein (FKBP) and FKBP-rapamycin binding (FRB), respectively. (B) The N-terminal domain of the dynein adaptor bicDN (amino acids 1 to 594) was recruited to the membrane anchor CAAX in (C) and (D). (C and D) Neurons expressed FRB-Cerulean3-CAAX for control, and with bicDN additionally tdTomato-bicDN-FKBP. Neurons were imaged for 20 to 40 min, after which 0.5 μ M rapalog A/C Dimerizer was added, and imaging continued. (C) Average MT-RF before and 6 min to 1 hour after dimerization was calculated from CAMSAP3 traces (for bicDN, $n = 28$ cells, $N = 5$; for control, $n = 49$, $N = 3$). (D) CAMSAP3 intensity along neurites (x axis) over time (y axis; kymograph). Dashed lines show the time at which the dimerizer was added. (E) Wild-type or adenosine triphosphatase (ATPase)-deficient mutated (K2599T) dynein1 motor domain (Dync1h1motor; amino acids 1453 to 4644) was recruited to the membrane anchor C2 for (F) and (G). (F and G) Neurons expressed FRB-C2-Cerulean3 with FKBP-mScarlet-Dync1h1motor (wild type) or FKBP-mScarlet-Dync1h1motorK2599T (motor deficient). Neurons were imaged for 1 to 2 hours, and then 0.5 μ M rapalog A/C Dimerizer was added before continuing imaging. (F) Average MT-RF was calculated before and 6 min to 1 hour after dimerization from CAMSAP3 traces ($n = 23$ cells for wild-type dynein motor, $n = 22$ cells for motor-deficient dynein motor, $N = 5$ independent experiments). (G) CAMSAP3 intensity along neurites (x axis) over time (y axis; kymograph). Dashed lines show the time at which the dimerizer was added. ** $P < 0.01$ and *** $P < 0.001$, Kruskal-Wallis multiple comparison with Dunn's post hoc test with Bonferroni correction.

taken together, the discovery of MT-RF opens a new field of potential research, in which there is much scope for fundamental discoveries.

Regulation of microtubule density cycles by MT-RF

During neuronal development, microtubule density in neurites constantly cycles between low and high density, with high-density states associated with increased neurite growth (27). We propose that these density cycles are caused by a balance between MT-RF and microtubule polymerization. Microtubules in the neurite polymerize more than 20 times faster than the retrograde flow of microtubules (14, 15). Microtubule polymerization of tubulin quickly diffusing or being transported into the neurite thereby compensates for the loss of microtubules through MT-RF. Because only some microtubules polymerize at any given time in the neurite, MT-RF and microtubule polymerization remain in a balance and keep microtubule density relatively constant. It is known that microtubule polymerization in neurites is increased in bursts during early neuronal development (27). When microtubule polymerization is increased, more microtubules polymerize than are lost from the neurite due to MT-RF; as a result, microtubule density increases. After this increase, microtubule polymerization decreases again and MT-RF, in turn, returns microtubule density back to baseline.

While previous models only explained the increase in microtubule density, this new model comprising MT-RF provides an explanation for the whole density cycle, including the decrease in microtubule density. This provides a basis for our understanding of neurite growth and could help pinpoint how neurites are able to explore space in search of chemical gradients for chemotaxis or connecting with neuronal and non-neuronal cells.

MT-RF and its role during neuronal polarization

Whereas previously the tip of the neurite was thought to regulate neurite growth (10, 52), our data uncover that the advance of the neurite tip is regulated by dynamics of the entire neurite, through MT-RF. We propose that MT-RF mechanically destabilizes the microtubule array to inhibit stable growth during the fluctuating state (9, 26). Slowdown of MT-RF in the axon then enables the stable axonal microtubule array to be used as a scaffold for extension. Such a mechanically stable microtubule array could be required for neurite growth during neuronal polarization, as neurites of central nervous system neurons can extend without using the matrix as a scaffold on which to pull (24). Consequently, our work provides a paradigm shift in our understanding of neuronal polarization. While previous studies focused mainly on the axon and its growth

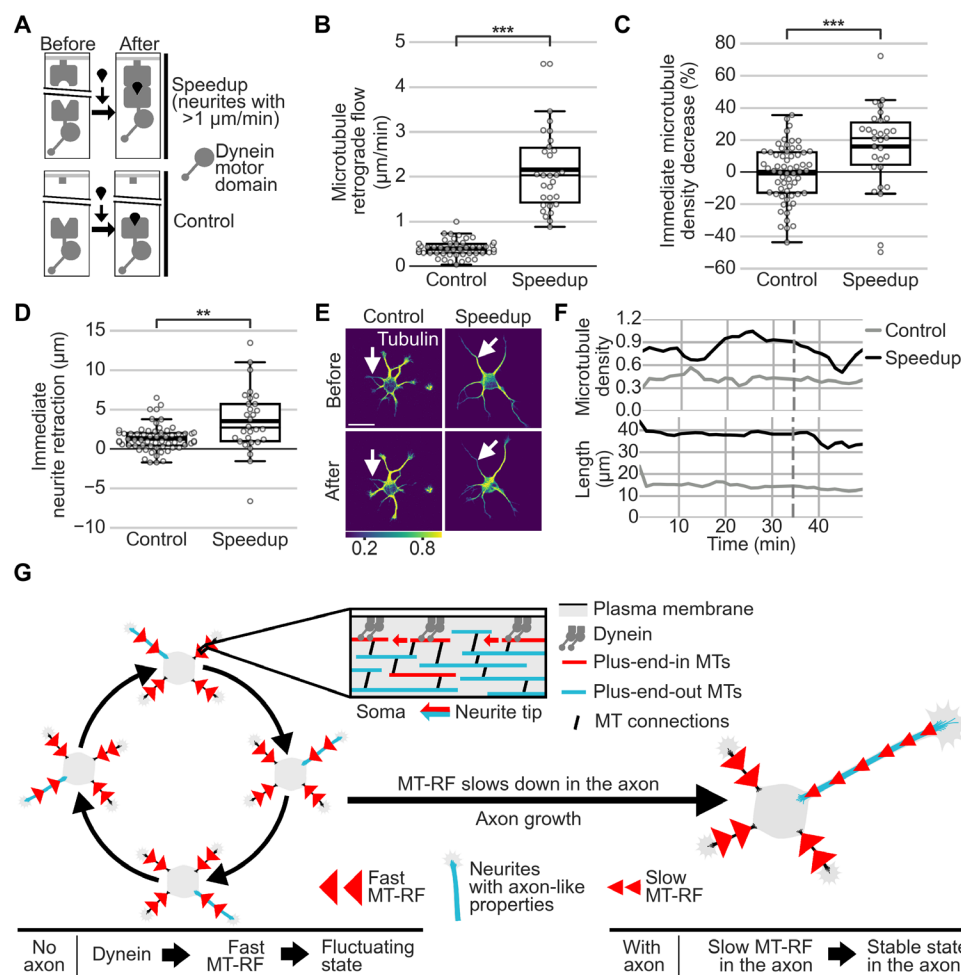


Fig. 10. Dynein at the plasma membrane induces immediate MT density decrease and retraction. Neurons expressed the fluorophore mNeonGreen fused to CAMSAP3 and were imaged after 1 day in culture. (A) The wild-type motor domain of dynein1 was recruited to the membrane anchor C2 for (B) to (F). (B to F) Neurons expressed FRB-C2-Cerulean3, FKBP-Halo-Dync1h1motor, and TUBB2a-mScarlet. Neurons were imaged for 20 to 40 min, and then 62.5 nM rapalog A/C Dimerizer was added before continuing imaging. (B) MT-RF was measured from CAMSAP3 traces by averaging MT-RF from 1.5 to 15 min after dimerization. To obtain neurites with speedup from neurons with membrane-recruited dynein motor, neurites with MT-RF faster than 1 $\mu\text{m}/\text{min}$ were used, while control neurites were analyzed independent of their MT-RF. Speedup was observed for membrane-recruited dynein in 86 of 368 neurites, but in 0 of 243 neurites for control. For control in (C) to (F), $n = 62$ cells; of those for (B), $n = 48$ cells, $N = 6$; for speedup, $n = 30$ cells, $N = 5$. (C) Immediate MT density decrease was calculated as the difference in normalized microtubule intensity in one neurite from before dimerization to the lowest intensity 6 to 12 min after dimerization. (D) Retraction was calculated as the difference in length of one neurite before dimerization to the smallest length 6 to 18 min after dimerization. (E) Morphology of representative cells with the neurites shown in (F) indicated by arrows. (F) Normalized tubulin intensity and length of representative neurites. (G) Illustration of MT-RF fueling fluctuations and then MT-RF slowing down in the axon. The thick line in boxplots shows mean. $**P < 0.01$ and $***P < 0.001$, Dunn's test. Scale bar, 20 μm .

properties (10, 11), MT-RF shifts this view toward the minor neurites and that maintaining a short length is an active process.

MT-RF constitutes a previously unknown type of cytoskeletal dynamics, synchronously restructuring the cytoskeleton on an unprecedented scale by driving continuous removal of the entire microtubule mass. To maintain microtubule mass, microtubules nucleate and polymerize within the neurite (4, 5). It will be interesting to see whether MT-RF also influences dynamics of microtubule-filled processes in invading carcinoma cells (53) and in other non-neuronal cells, including radial glia cells, developing oligodendrocytes or microglia during development, maintenance, and disease. Decades ago, the discovery of actin filaments treadmilling spawned the concept of actin retrograde flow (54–56) that radically changed the study of

cytoskeleton dynamics in neurons (52) and non-neuronal cells (57). As the discovery of MT-RF is a comparable conceptual shift, we anticipate a similar boost in research.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the Animal Welfare Act and the guidelines of the North Rhine-Westphalia State Environment Agency [Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV); reference number for approval (Aktenzeichen: 84-02.04.2015.A334 and Aktenzeichen 81-02.04.2021. A208)]. Animals were group-housed (up to five mice per cage) with

room temperature controlled at 21° to 22°C and an artificial 12-hour light:12-hour dark cycle (lights off at 6:00 p.m.). Mice (adult C57Bl6/J, Charles River Laboratories, JAX C57BL/6J; RRID: IMSR_JAX:000664) were given food and water ad libitum throughout the experiment.

Neuron culture

Primary hippocampal neurons were obtained from embryonic day 16.5 (E16.5) to E17.5 mouse brains. Cells were kept at 36.5°C and 5% CO₂. Neurons were cultured without a glia feeding layer, as previously described (58). After dissection, hippocampi were collected in Hanks' balanced salt solution with magnesium and calcium (Thermo Fisher Scientific). Next, hippocampi were digested in 0.25% trypsin (Thermo Fisher Scientific, 25-200-056) at 37°C for 15 min and subsequently washed three times with minimum essential medium (MEM)–1% horse serum (MEM–1% HS) medium [1× MEM, 1× essential and nonessential amino acids, 2 mM L-glutamine (all from Thermo Fisher Scientific), 0.22% NaHCO₃, 0.6% glucose, and 10% HS (Pan-Biotech, P30-0702)]. Last, hippocampi were mechanically dissociated with fire-polished glass-Pasteur pipettes. After transfection, 10,000 or 15,000 neurons were plated in eight-well glass-bottom dishes (ibidi, 80827-90). Dishes were coated with poly-L-lysine (1 mg/ml) at room temperature overnight, three to four times washed with double-distilled water, and placed in the incubator at 36.5°C and 5% CO₂ with MEM–10% HS to equilibrate for at least 2 hours before plating. Two to 4 hours after plating neurons, MEM–10% HS medium was replaced by neuronal medium (N2) [1× MEM, 1 mM sodium pyruvate, 1% Neuropan 2 supplement (Pan-Biotech, P07-11010), NaHCO₃, 0.6% glucose, and 2 mM L-glutamine], which was preconditioned through 2 to 3 days of incubation with a glia feeder layer.

Neuron transfections

Cell transfections were performed using Nucleofector II (Lonza, AAB-1001) with program 0-005 and the mouse neuron Nucleofector Kit (Lonza, VPG-1001) according to the manufacturer's specifications. For each transfection, 5×10^5 neurons were used with varying amounts for different plasmids (table S2): 15 µg of pBetaActin-TUBB2a-mEos3.2, 15 µg of pBetaActin-TUBB2a-Dronpa, 6 µg (Fig. 3) or 3 µg (all other figures) of pBetaActin-mNeonGreen-CAMSAP3, 10 µg of pBetaActin-tandem-mCherry, 10 µg of pBetaActin-TUBB2a-mNeonGreen, 10 µg of pBetaActin-TUBB2a-mScarlet and 10 µg of pHalo-N1-TUBB5, 3 to 6 µg of pBetaActin-IC2N-dCER3, 8 µg of pBa-tdTomato-bicDN-FKBP, 10 µg of pBetaActin-FRB-dCER3-CAAX, 10 µg of pBetaActin-FRB-C2-dCER3, 10 µg of pBetaActin-dCER3-C2-dCER3, 2 µg of pBetaActin-Halo-CAMSAP3, 12 µg of pBetaActin-FKBP-mScarlet-Dync1h1motor, 12 µg of pBetaActin-FKBP-mScarlet-Dync1h1motorK2599T, 12 µg of pBetaActin-FKBP-Dync1h1motor, 12 µg of pBetaActin-FKBP-Halo-Dync1h1motor, 8 µg of pBetaActin-FKBP-mScarlet-KIF1Cmotor, 8 µg of pBetaActin-FKBP-mScarlet-KIF1CmotorN593K, 1 µg of pBetaActin-EB3-mNeonGreen, 10 µg of pBetaActin-KIF1aMotor-mScarlet-FKBP, and 10 µg of pBetaActin-KIF13aMotor-mScarlet-FKBP. All plasmids were prepared with the EndoFree Maxiprep Kit (QIAGEN).

Collagen 3D matrices

Neurons were grown in 3D collagen matrices as described previously at a density of 0.75×10^6 to 1.5×10^6 cells/ml (24). For the collagen matrix, 225 µl of collagen (Merck Millipore, catalog no. 08-155) with a concentration of 4.13 mg/ml was combined with 30 µl of 10× MEM and 15 µl of 5.5% sodium bicarbonate and mixed. Transfected cells

in medium were added 1:3 and mixed carefully. From the hydrogel-cell mix, single 40-µl drops were added to the bottom of eight-well glass-bottom dishes (ibidi). Gels were kept in a cell culture incubator at 37°C for 20 min before the addition of 400 µl of conditioned N2 medium to fully cover gels.

Ex utero electroporation and organotypic slice culture of embryonic brain

Ex utero electroporation and organotypic brain slices were prepared as previously described (59). Briefly, cortices of E14.5 embryos were electroporated with pBetaActin-TUBB2a-mEos3.2-mEos3.2 (2 µg/µl) (10% FastGreen solution) by injecting DNA in both lateral ventricles using glass capillaries (prepared with a capillary electrode puller with the following settings: pressure: 500; heat: 800; pull: 30; velocity: 40) and then electroporating at an approximately 60° angle with the cathode facing the cortex (five pulses, duration of 50 ms, 30 mV, 1-s interval). Embryonic brains were then embedded in 3% low-melt melting point agarose (Biozyme, Biozym Sieve GP Agarose, 850080), and coronary slices with 180 µm thickness were prepared using a Vibratome (Leica VT1200 S vibratome). Slices were kept on polytetrafluoroethylene (PTFE) membranes (30 mm, hydrophilic PTFE, 0.4 µm; EMD Millipore, PICM0RG50) in 35-mm dishes with 1-ml slice medium (Neurobasal 1× 5% fetal calf serum, B27 supplement 1:50, GlutaMAX 1:400, penicillin-streptomycin 1:200, all from Thermo Fisher Scientific; 5% HS and Neuropan 2 supplement 1:100 from Pan-Biotech) at 35°C, 5% CO₂ for 2 days. Directly before imaging, slices were carefully transferred into eight-well glass-bottom ibidi dishes.

DNA constructs

pBa-tdTomato-flag-BicD2 594-FKBP (pBa-tdTomato-bicDN-FKBP) was a gift from G. Banker and M. Bentley (Addgene, plasmid no. 64205; <http://n2t.net/addgene:64205>; RRID: Addgene_64205) (60). pHalo-N1-TUBB5-Halo was a gift from Y. Okada (Addgene, plasmid no. 64691; <http://n2t.net/addgene:64691>; RRID: Addgene_64691) (13). pBetaActin-tandem-mCherry was a gift from S. Dupraz German Center for Neurodegenerative Diseases (DZNE). pBetaActin-EB3-mNeonGreen was a gift from S. Vinopal [Jan Evangelista Purkyně University in Ústí nad Labem (UJEP)].

All DNA plasmids made in this study were constructed in the pBetaActin plasmid backbone [gift from C. C. Hoogenraad (University Utrecht)] (34). Plasmids were constructed using homology-based cloning with the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, #E2621L) according to the manufacturer's specifications. Protein coding sequences were assembled by polymerase chain reaction (PCR) amplification using Q5 polymerase (New England Biolabs, #M0491S), using primers with homology sequences in overhanging ends and subsequent cleanup from an agarose gel. The plasmid backbone for these assemblies was constructed by digestion of pBetaActin plasmid with Nhe I and Hind III and subsequent cleanup from an agarose gel. One sequence was designed as a homology site for the upstream sequence in the backbone (pBetaActin upstream: CTCTGTTCTCCGACGCCCCCAAGCTAGC), two similar sequences for the downstream sequence (pBetaActin downstream 1: CGACTGCAGAATTGCAAGCTTCTAGA and pBetaActin downstream 2: GGTACCGTTCGACTGCAGAATTGCAAGCTTTTA), and two sequences to combine protein-coding sequences together (linker 1, GGAAGTTCAGGAGGTTCTAGT, will be translated into the neutral linker sequence GSSGSS; linker 2, TCTGGTGGATCAAGT-GGTGGA, will be translated into the neutral linker sequence SGGSSG).

To combine two protein-coding sequences, only linker 1 was used, while to combine three protein-coding sequences, linker 1 was used between the first and second protein-coding sequences and linker 2 between the second and third protein-coding sequences. The same homology sequences were used for all assemblies. Primer sequences for all PCR products used for cloning plasmids can be found in table S1. The protein-coding sequence of TUBB2a was amplified from mouse cDNA. mEos3.2 was amplified from the plasmid mEos3.2-N1 (gift from M. Davidson; Addgene, plasmid no. 54525; <http://n2t.net/addgene:54525>; RRID: Addgene_54525) (13). Dronpa was amplified from the plasmid Dronpa-Tubulin-6 (gift from M. Davidson; Addgene, plasmid no. 57301; <http://n2t.net/addgene:57301>; RRID: Addgene_57301). CAMSAP3 was amplified from pFasBac+GFP-CAMSAP3 (gift from R. Vale; Addgene, plasmid no. 59038; <http://n2t.net/addgene:59038>; RRID: Addgene_59038) (61). MNeonGreen was amplified from the pNCS-mNeonGreen plasmid (Allele Biotechnology). MScarlet was amplified from pLifeAct_mScarlet_N1 (gift from D. Gadella; Addgene, plasmid no. 85054; <http://n2t.net/addgene:85054>; RRID: Addgene_85054) (13). pHalo-N1-TUBB5-Halo was a gift from Y. Okada (Addgene, plasmid no. 64691; <http://n2t.net/addgene:64691>; RRID: Addgene_64691) (13). C2 was amplified from CRY2clust-eGFP-2xC2-iLID (gift from D. Gadella; Addgene, plasmid no. 114420; <http://n2t.net/addgene:114420>; RRID: Addgene_114420) (62). IC2N was amplified from EGFP-IC2-N237 (gift from T. Schroer; Addgene, plasmid no. 51410; <http://n2t.net/addgene:51410>; RRID: Addgene_51410) (31). Cerulean3 was amplified from pTriEx-DORA (gift from Y. Wu) (63). CAAX was amplified from pLL7.0: Venus-iLID-CAAX (from KRas4B) (gift from B. Kuhlman; Addgene, plasmid no. 60411; <http://n2t.net/addgene:60411>; RRID: Addgene_60411) (64). FRB was amplified from pBa-FRB-3myc-KIF1A tail 391-1698 (gift from G. Banker and M. Bentley; Addgene, plasmid no. 64286; <http://n2t.net/addgene:64286>; RRID: Addgene_64286) (60). FK506-binding protein (FKBP) was amplified from pBa-tdTomato-flag-BicD2 594-FKBP (gift from G. Banker and M. Bentley; Addgene, plasmid no. 64205; <http://n2t.net/addgene:64205>; RRID: Addgene_64205) (60). Dync1h1 motor domain (heavy chain 1 amino acids 1453 to 4644, Dync1h1motor) was amplified from mouse cerebrum cDNA. To generate the mutated motor-deficient dynein motor domain, the correct mutation site was identified by homology analysis of protein-coding sequence for *Dictyostelium* Dynein1 and mouse Dync1h1. Mutation in *Dictyostelium* dynein1 K2675T corresponded to mouse Dync1h1 K2599. To perform the mutation, primers containing AAG to ACG mutation were designed to amplify Dync1h1motor as downstream and upstream parts, each extending 10 to 20 nucleotides over the mutation to have 20 nucleotides overlap between the two amplicons. The corresponding PCR products were used in the usual assembly strategy to directly clone pBetaActin-FKBP-mScarlet-Dync1h1motorK2599T. The mouse KIF1A motor domain (amino acids 1 to 405) was amplified from mCh-KIF1a*-strep (gift from J. Bonifacino; Addgene, plasmid no. 120165; <http://n2t.net/addgene:120165>; RRID: Addgene_120165) (36). The human KIF13A motor domain (amino acids 1 to 639) was amplified from mCh-KIF13a*-strep (gift from J. Bonifacino; Addgene, plasmid no. 120166; <http://n2t.net/addgene:120166>; RRID: Addgene_120166) (36). The human KIFC1 motor domain (amino acids 125 to 673) was amplified from Strep-KIFC1*-mCh (gift from J. Bonifacino; Addgene, plasmid no. 120169; <http://n2t.net/addgene:120169>; RRID: Addgene_120169) (36). To generate the motor-deficient KIFC1 motor domain mutant N593K (38), primers containing AAG to AAC mutation were designed to

amplify KIFC1 as downstream and upstream parts, each extending 10 to 20 nucleotides over the mutation, so that the two amplicons had 20 nucleotides overlap between each other. The corresponding PCR products were used in the usual assembly strategy to directly clone pBetaActin-FKBP-mScarlet-KIFC1motorN593K.

Live-cell imaging

All imaging experiments were performed on an Andor spinning disk Nikon Eclipse Ti microscope with Perfect Focus system (Nikon) and a Yokogawa CSU-X1 spinning disk unit (CSUX1-A1N-E/FB2 5000 rpm Control, FW, DMB 95L100016) using a Plan Fluor VC 40× oil objective numerical aperture (NA) 1.3 (Fig. 5, B to E, and fig. S12), Plan Apo VC 60× water objective NA 1.2 (Nikon; Fig. 2, E and F, and fig. S6), or Plan Apo VC 60× oil objective NA 1.4 with an additional 1.5 magnifier (figs. S1 and S15) or no additional magnifier (Nikon; all other figures and supplementary figures) and iQ3 software (Andor). A dichroic Quad filter (for wavelengths: 395 to 410, 482 to 492, 561 to 568, and 628 to 650) and a single band-pass emission filter for the blue channel (480/40; Semrock), the green channel (525/50; Semrock BrightLine), the red channel (617/73; Semrock BrightLine), and the far-red channel (697/75; Semrock BrightLine) were used. For most photoconversion experiments (Figs. 1, B and C, 2, B and C, 4, C and D, 6, B and C, and 8, B to E, and figs. S2, S3, S5 to S8, and S12), a quad band emission filter was used (440/40, 521/21, 607/34, and 700/45; Semrock Quad Band Filter FF01-440/521/607/700) to allow faster imaging without changing filters. For the green, red, and far-red channels, a 488-nm laser (100 mW), a 561-nm laser (100 mW), and a 640-nm laser (100 mW) were used, respectively (REVOLUTION 500 series AOTF laser modulator and combiner unit, solid-state laser modules). Experiments were conducted inside a large incubator chamber around the microscope, kept at 37°C. A small chamber for eight-well ibidi glass-bottom dishes (Pecon, #000470) was supplied with humidified air mixed with CO₂ to a concentration of 6 to 7% CO₂. Images were taken with an Andor iXON camera (DU897) in charge-coupled device mode at 1-MHz horizontal readout rate and with a pixel size of 0.44 μm (2 × 2 binning; Figs. 1, B and C, 2, B and C, 4, C and D, 6, B and C, and 8, B to E, and figs. S2, S3, and S5 to S8), 0.22 μm (no binning; Figs. 1, E and F, 3, B to E, 7, B to D, 9, C, D, F, and G, and 10, B to F, and figs. S4, S11, S13, S14, and S16), 0.15 μm (no binning; 1.5× magnifier; figs. S1 and S15), or 0.33 μm (no binning; 40× objective; Fig. 5, B to E, and fig. S12).

Images were either taken every 60 s (Figs. 3, B to E, 5, B to H, and 9, C and D), every 3 min (Fig. 7, B to D), every 90 s (Figs. 9, F and G, and 10, B to F, and figs. S11 to S14), every 20 s (fig. S16), every second (fig. S15), or as specified in the “Photoconversion and photoactivation” section.

For all experiments, neurons were selected on the basis of representative morphology of more than two neurites. Imaging was done under the lowest possible light exposure. Long exposure times (800 to 1000 ms) and lower laser power were preferred over shorter exposure times and higher laser power.

For drug treatments in Figs. 6 and 7 and fig. S15, 40 μM pa-Blebb [stock in dimethyl sulfoxide (DMSO); Axol Bioscience, ax494682] (65), 6 nM taxol (stock in DMSO; Biomol, Cay10461-25), 10 μM LatB (only for fig. S15; stock in DMSO; Merck Millipore, 428020), or DMSO (Sigma-Aldrich, D5879) as a control (all to a final DMSO concentration of 0.2%) was used. For fig. S4, 0.75 μM LatA (stock in EtOH; Sigma-Aldrich, 428021) or EtOH as a control was used. For Figs. 6 and 7, drugs were added to cells after 1 day in culture for 20 to 240 min directly before the experiment. For fig. S4, LatA or EtOH

was added 240 min before imaging. For fig. S14, 10 μM LatB was added 4 to 8 hours before imaging. For fig. S15, drugs were added 3.5 to 7 hours before imaging. For drug treatments in Fig. 8 (B to E), 50 μM ciliobrevin A (Sigma-Aldrich, HPI-4, H4541) or DMSO as control was added 30 to 60 min to cells before imaging started.

Dimerization experiments

For dimerization experiments (Figs. 9, C, D, F, and G, and 10, B to F, and figs. S11 to S14 and S16), 0.5 μM (final EtOH concentration of 0.1%; Fig. 9, C, D, F, and G, and fig. S13) or 62.5 nM (0.0125% final EtOH concentration; Fig. 10, B to F, and figs. S11, S12, S14, and S16) of the rapalog A/C Dimerizer (SiChem, SC-7000) was added after imaging for 20 to 90 min, with imaging continued after dimerization for 30 to 300 min. Images were taken every 20 s for imaging neurons with recruitment of the KIF motor domain (fig. S16) and 60 or 90 s for all other dimerization experiments.

Photoconversion and photoactivation

For photoconversion and photoactivation experiments, the fluorescence recovery after photobleaching and photoactivation (FRAPPA) illumination system was used (Andor, Revolution). The FRAPPA module focuses the laser on single pixels for a specified time (dwell time) and moves through all pixels for a specified number of repetitions. A small area for photoconversion was chosen (specific sizes for experiments described below) to keep phototoxicity minimal.

In experiments with photoconversion of microtubule patches (Figs. 1, B and C, 2, B and C, 4, C and D, 6, B and C, and 8, B to E, and figs. S2 and S8), small microtubule patches were photoconverted in two neurites. For fig. S5, two microtubule patches were photoconverted in one axon, and for fig. S6, one microtubule patch was converted in one dendrite and one in the axon. Photoconversion was performed with the 405-nm laser (400 repetitions, 20 μs dwell time, 0.37 μW power, pixel size of 0.44 μm , 2-pixel $\times$ 1-pixel or 1-pixel $\times$ 1-pixel area). After photoconversion, images were acquired in the photoconverted (red) and not-photoconverted (green) channel every 30 s for 20 min (fig. S4) or 10 min (Figs. 1, B and C, 4, B and C, 6, B and C, and 8, B to E, and figs. S2, S3, S5, S7, and S8) or every 90 s (figs. S11, S12, and S14). For most experiments in Figs. 1 (B and C) and 2 (B and C), images of all cells were taken again 1 to 3 hours after photoconversion to check whether cells survived the experiment. Cells that died were excluded from the analysis.

For experiments with photoconversions after dynein motor domain recruitment to the plasma membrane (figs. S11, S12, and S14), photoconversion was performed with the 405-nm laser (800 repetitions, 20 μs dwell time, 0.37 μW power, pixel size of 0.22 μm , 6-pixel to 12-pixel $\times$ 1-pixel area).

In experiments with photoconversion of microtubule patches after dynein motor domain recruitment to the plasma membrane in young neurons without LatB treatment (fig. S11), CAMSAP3 was measured in addition to photoconverted microtubule patches. Microtubule patches were repeatedly photoconverted with varying time intervals once the start of MT-RF speedup in the CAMSAP3 channel was visible by eye. In LatB-treated neurons with dynein recruitment (fig. S14), photoconversion was performed 20 to 60 min before dimerization and directly after dimerization. For photoconverting microtubule patches in experiments recruiting the dynein motor domain to the plasma membrane in neurons cultured for 7 days (fig. S12), photoconversions were performed 12 to 40 min before dimerization and then repeated directly after dimerization.

In experiments with photoconversion of microtubule patches in neurons grown in 3D collagen matrices (fig. S6), photoconversion was done with the 405-nm laser (0.5 μW power, 400 repetitions, 20 μs dwell time, 2-pixel $\times$ 1-pixel area, 0.44 μm pixel size). After photoconversion, images were taken every 2 min for 10 min. Z-stacks were acquired at a z-step size from 1.7 to 1.85 μm . To mitigate the effect of retraction on measured MT-RF, we excluded neurons that showed noticeable neurite retraction. To check whether neurites retracted, the entire neuron was imaged as a larger z-stack following the experiment.

For photoconversion of neurons in organotypic brain slices (Fig. 2, E and F), photoconversion was done with the 405-nm laser (0.5 μW power, 400 repetitions, 20 μs dwell time, a 6-pixel to 10-pixel $\times$ 1-pixel area, 0.22 μm pixel size). Images were taken at an interval of 3 min for 30 min after photoconversion. Z-stacks were acquired at a z-step size of around 1 μm .

To test whether photoconversion reaches the entire microtubule stack (fig. S1), all neurites were photoconverted with the 405-nm laser (0.37 μW power, 400 repetitions, 20 μs dwell time, 2-pixel $\times$ 1-pixel area, 0.44 μm pixel size). Z-stacks were acquired with a z-step size of 0.25 to 0.26 μm .

In experiments where microtubule patches were photoactivated (Fig. 1, E and F, and fig. S4), two or three microtubule patches were photoactivated in a single neurite. Before photoactivation, the entire cell was bleached by taking a brief 200-ms image with high power (50%) with the 488-nm laser. Photoactivation was done with the 405-nm laser with less than 1% of the total light intensity required for photoconversion of mEos3.2 (0.04 μW power, 40 repetitions, 20 μs dwell time, 4-pixel $\times$ 1-pixel area, 0.22 μm pixel size). After photoactivation, one image was taken every 1 min for 6 min. During imaging, Dronpa gradually recovered fluorescence. To prevent background intensity from becoming too high, before every acquired frame, a part of the soma was bleached with the 488-nm laser (3.51 μW power, 10 repetitions, 20 μs dwell time, 10-pixel $\times$ 12-pixel area, 0.22 μm pixel size).

Analysis of photoconversion in whole microtubule stack

For fig. S1, chromatic shift was corrected between the red (TUBB2a-mEos3.2; photoconverted tubulin) and the far-red channel (TUBB5-Halo; whole tubulin). The intensity in the red channel was higher than in the far-red channel when further from the cover glass. Both channels were first background subtracted. The chromatic shift was determined by measuring which z-slice number had the highest intensity in each channel and averaging the difference between these z-slice numbers across all neurites (around 1.8 z-slices, which was rounded to 2 z-slices). The average intensity for all neurites in both channels was measured in ImageJ after manual thresholding both channels to exclude neurite background. Average intensities were normalized to the highest intensity in the respective region and channel. The normalized intensities of three slices below and above the maximum slice were averaged for each channel and region. The slices with the highest normalized intensity were excluded from analysis because they are always “1.”

Imaging of EB3 for microtubule orientation

For imaging EB3 comets to measure microtubule orientation, neurons expressing EB3-mNeonGreen were imaged once a second for 60 s before treatment. After 3.5 to 7 hours of treatment (DMSO as control, 40 μM pa-Blebb, 6 nM taxol, or 10 μM LatB), neurons were imaged again for 60 s once a second. For taxol treatment, cells were

washed three times with taxol-free medium approximately 15 min before images.

Registering low-intensity time-lapse movies (Python script *livecellreg*)

Time-lapse movies—used to generate kymographs (Figs. 3, B to E, 5, B to E, 7, B to D, and 9, C, D, F, and G, and 10, B to F, and figs. S11 to S14 and S16) or used for measuring MT-RF in brain slices (Fig. 2, E and F)—were registered by correcting for translation shifts with a self-made Python script. The script was developed because standard ImageJ registering plugins tested [“StackReg” (https://github.com/fiji-BIG/StackReg/blob/master/src/main/java/StackReg_.java) and “Register Virtual Stack Slices” (<https://imagej.net/plugins/register-virtual-stack-slices>)] did not yield satisfying results, likely because of the low intensity of registered channels. The script can register by translation in two or three dimensions. Before starting time-lapse movie registration, the maximum intensity of the movie in each pixel was set as 6 to 7 SDs of the image intensity from all time frames above the average image intensity from all time frames. This also allowed reliable registration of signals that markedly changed localization within the cell (e.g., CAMSAP3 after dynein motor domain recruitment to the membrane). To register a time-lapse movie, the correlation of a reference image with that of the frame to be registered was calculated to one pixel shift for each dimension, for eight shifts in total. The radius of this analysis successively increased by one pixel, and the direction of the shift with the best correlation value with regard to the starting shift was determined. This process was stopped when one direction of the best correlation value was obtained five times. The shift position with the best correlation value until then was used as a starting point to refine the shift values. For each dimension (x , y , and z) successively, the direction in which the correlation value improved was tested. If correlation improved in one direction, the shift was increased in that direction in steps of one pixel until the correlation value did not improve anymore for a defined number of pixels. This was repeated for each dimension until, at one shift value, no improvement in any dimension was found. Best shift values in x and y were used as starting shift values for the next frame; to account for gradual shifts in time-lapse movies, images were shifted by rolling them in a shift direction (`numpy.roll`). Reference images were rolled to avoid problems with zero values when registering low-intensity images. The reference image was changed if the correlation value using the shift from the last time frame was at least 2 SDs different from the average correlation value obtained for time points until then. This often indicated a morphological change of the neuron, which made a new reference image advantageous as it was morphologically closer to the images afterward. To register time-lapse movies from neurons in brain slices, a new reference image was additionally defined every three frames. Registered images were saved with zero values instead of rolled area in shifted areas to make the shift clearly visible. The script is available via GitHub (<https://github.com/maxschelski/livecellreg>).

Kymograph generation

Time-lapse movies from which kymographs were generated were registered with a self-made Python script (see the previous section). Kymographs were generated manually in ImageJ using the KymoResliceWide plugin (<https://imagej.net/plugins/kymoreslicewide>) and a multiline tool with a line width of 11 to 25 pixels (Figs. 9, C, D, F, and G, and 10, B to F, and fig. S13), 11 to 40 pixels (figs. S11,

S12, S14, and S16), or 11 pixels (Figs. 3, B to E, 5, B to E, and 7, B to D) and maximum intensity extracted across that width. Because of large changes in dendrite morphology after dynein motor membrane recruitment in some neurons from fig. S12, multiple kymographs were drawn over time to always cover the entire dendrite length. These kymographs were combined afterward to get one kymograph for the entire length of the experiment. Lines were oriented so that the left of the kymographs corresponded to the direction of the soma and the right to the direction of the neurite tip.

Automated analysis of neurite length from kymographs

For Figs. 5 (B to E), 9 (F and G), and 10 (B to F) and figs. S2, S14, and S16, neurite length was automatically analyzed from kymographs using a self-made Python script. In these kymographs, rows from top to bottom corresponded to increasing time points in the time-lapse movie, while columns from left to right corresponded to points along the neurite from soma to neurite tip. For Figs. 5 (B to E) and 9 (F and G) and fig. S2, first, kymographs were smoothed (`scipy.ndimage.median_filter`, `size = 2`). For Fig. 10 (B to F) and figs. S14 and S16, kymographs were smoothed across rows [position in neurite; `scipy.ndimage.median_filter`, `size = (1,5)`] to prevent smoothening across time points (columns). Kymographs were then thresholded using a value determined manually. In thresholded kymographs, the first and last time points were always added to the thresholded area to allow for binary holes (`scikit-image.morphology.binary_fill_holes`). For Figs. 5 (B to E) and 9 (F and G), the thresholded image was dilated before filling holes (`scipy.ndimage.binary_dilation`, `size = 2`). The resulting area only included the neurite. Neurite length was determined for each time point as the distance from the soma to the pixel furthest from the soma that still belongs to the neurite. The last pixel that belongs to the neurite was defined as one pixel before the first not thresholded pixel closest to the soma.

Classification of axon formation

Neurons were classified as lacking an axon when the longest neurite was shorter than 30 μm or under 5 μm longer than the second-longest neurites, as well as shorter than 50 μm . For fig. S8, neurons were considered to be at the axon transition if the longest neurite was at least 30 μm long and at least 5 μm longer than the second-longest neurite. Neurons were classified as having an axon if the longest neurite was at least 50 μm long and 10 μm longer than the second-longest neurite.

Analysis of MT-RF

To measure MT-RF in 2D after tubulin photoconversion (Figs. 1, B and C, 2, B and C, and 4, B and C, and figs. S5, S7, and S12) or photoactivation (figs. S3 and S4), movement of microtubule patches was traced. The midpoint of microtubule patches was visually estimated, and movement along the neurite over time was traced in ImageJ using the multiline tool. Only microtubule patches that could be traced for more than 1 min were analyzed.

To analyze MT-RF in neurons without axons together with neurite growth (fig. S2), the maximum intensity along neurites (x axis) was plotted against time (y axis; kymograph). Image data from Fig. 1 (B and C) were used. Using the ImageJ line tool, the slope of the moving photoconverted microtubule patch in the kymograph was measured by dividing the width (distance) through the height (time). Length was analyzed automatically from kymographs generated from the nonphotoconverted channel, as described in the section above

(see the “Automated analysis of neurite length from kymographs” section). For Fig. 1 (B and C), one neurite was excluded from analysis (speed of 3.7 $\mu\text{m}/\text{min}$) because its speed was 70% higher than the second-highest value and more than 8 SDs away from the mean.

To analyze movement of multiple microtubule patches in the same neurite (Fig. 1, D to F), movement of two to three microtubule patches in the same neurite was traced, using the same procedure as for photoconverted microtubule patches. For all combinations (up to three patches), the difference in the traveled distance was calculated. The highest traveled difference was divided by the two lower distances to obtain the relative difference in traveled distance.

To analyze MT-RF in neurons cultured in 3D collagen matrices (fig. S6) and in neurons in brain slices (Fig. 2, E and F), the movement of microtubule patches across x , y , and z was traced. The midpoint of patches was located by eye and marked using the ImageJ point tool for different time points in the z -slice, with the highest intensity of the microtubule patch. These points were used to calculate the distance the microtubule patch moved.

For measuring MT-RF with fluorescently labeled CAMSAP3 (Figs. 3, B to E, 5, B to E, 9, C, D, F, and G, and 10, B to F, and figs. S11, S13, S14, and S16), kymographs were generated (see the “Kymograph generation” section) and CAMSAP3 was traced by using the ImageJ line tool. Speed was calculated as the width (distance) divided by the height (time) of the line, whereas the angle was used to calculate direction of CAMSAP3 signals. For all analyses, only retrogradely moving CAMSAP3 signals were considered. For each time point, MT-RF speed was calculated as the median speed of all traces. To analyze slowdown of MT-RF in the axon (Fig. 3, A to E), MT-RF was smoothed with a window size of 200 min. This allowed us to exclude moments when MT-RF in another neurite slowed down to the level of the axon. MT-RF was determined to have slowed once smoothed MT-RF was 20% slower in the axon compared to all other neurites for at least 180 min.

Fully automated script for neurite analysis (Python package “pyNeurite”)

We developed a Python package (pyNeurite; <https://github.com/maxschelski/pyneurite>) that traced and analyzed neurites in time-lapse movies. The package worked in multiple stages for each time frame: first, soma of the neuron was located and then the ideal threshold for the neuron was found. After that, neurites were skeletonized and traced. The last step was an analysis of intensities within neurites. The soma was extracted by using a grey opening algorithm (`scipy.ndimage.grey_opening`), edge extraction (`scikit-image.filters.scharr`), automated thresholding (`scikit-image.filters.threshold_otsu`), and then filling of the soma from the resultant edges (`scipy.ndimage.binary_fill_holes`). To obtain neuronal threshold, the lowest threshold value was found, which extracts the soma with the same size as the edge analysis. The threshold was then incrementally increased, and the thresholded image was skeletonized (`scikit-image.ndimage.skeletonize`). The final threshold was found a step before five or more pixels of the neurite skeleton were lost. Any gaps in neurites in the thresholded image—potentially introduced by thresholding—were closed by connecting segments detached from the soma back to soma once more. This was done by finding the point closest to the soma for each detached segment and connecting it to the closest point connected to the soma. Connections allowed were only the ones approximately in the same direction as detached segments, preventing these segments from being connected to a wrong nearby neurite, which may be closer.

The dilated thresholded soma (`scikit-image.morphology.binary_dilation`) was removed from the resulting image, followed by skeletonization to obtain neurite skeletons. For each neurite, branch points were identified, which separated different subbranches of a neurite. Subsequently, all branches of each neurite were constructed, with each branch starting from the soma. Terminal subbranches shorter than 1 μm and neurites with less than 40 points (9 to 15 μm) were excluded. Neurites with different starting points—but overlapping in different time frames—were excluded to prevent mistakenly traced neurites from being analyzed. In addition, different branches that overlapped 70% or more in different time frames were excluded. Tracing was most challenging when a neurite was crossed by another neurite because crossing points sometimes were interpreted as branching points. By only using branch points when intensities of emerging branches were within a similar range, this error was reduced. The script only worked well with markers that label the cytosol or neurites, and not when the neurite tip was predominantly labeled (e.g., for actin or F-actin). This package is available on GitHub (<https://github.com/maxschelski/pyneurite>).

Analyzing microtubule density cycles

For Fig. 5 (B to E), tubulin intensity was obtained from kymographs (see the “Kymograph generation” section) of the tubulin channel. To obtain average intensities of tubulin in neurites, kymographs were thresholded using the manually determined maximum background value. For each time point, the average intensity in the thresholded area was calculated. Instead of using kymographs for Fig. 7 (B to D), intensities were measured by a self-made fully automated Python script (see the “Fully automated script for neurite analysis (Python package “pyNeurite”)” section). For each neurite, only the branch present in most time frames was analyzed. Tubulin intensities were used (Figs. 5, B to E, and 7, B to D) to analyze microtubule density cycles using a self-made Python script. To analyze the frequency of microtubule density cycles (Figs. 5B and 7, B to D), intensity values were normalized by the average expression levels in all neurites. The average expression level was obtained by first averaging the intensity in all neurites for each time point. This average intensity over time was fitted to a quadratic function (Fig. 5, B to D) or a linear function (Fig. 7, B to D) using the `polyfit` function of the `numpy` package (`numpy.polyfit`) to obtain the average expression level. Intensity traces were smoothed using an averaging rolling window with a size of 2. Cycles were determined through changes of at least 0.2. One cycle consisted of an increase of 0.2 followed by a decrease of 0.2 or vice versa. The frequency of cycles was obtained by dividing the number of these cycles by the total time. For Fig. 5B, the frequency was averaged for all minor neurites of the neuron. For Fig. 7B, neurons with an axon were excluded and the frequency of microtubule density cycles was averaged for all neurites of each neuron. All speed values for one neurite were then averaged. For Fig. 5E, neurons were only considered before a neurite reached axon-like length. To correlate MT-RF with microtubule density cycles (Fig. 5E), Pearson’s r was calculated using the Python package `SciPy` (`scipy.stats.pearsonr`). Linear regression lines were plotted using the function `lmlplot` from the Python package `seaborn` (`seaborn`; <https://pypi.org/project/seaborn/>).

For Fig. 5 (B to E), the time of axon formation was determined by using neurite length that had been automatically measured from kymographs (see the “Automated analysis of neurite length from kymographs” section). As before, an axon was defined as being at least 50 μm long and at least 10 μm longer than the second-longest neurite.

Analysis of microtubule orientation (Python package “neuriteMT”)

Microtubule orientation was analyzed from polymerizing microtubule ends (fig. S15), labeled by expression of fluorescently tagged end-binding protein 3 (EB3). Live-cell imaging movies of EB3 were then automatically processed using the “plusTipTracker” of “u-track 2.0” in Matlab (66). For comet detection, a low-Gaussian SD of 1 pixel, high-pass Gaussian SD of 4 pixels, and a minimum threshold of 3 SDs with a threshold step size of 1 SD were used. For tracking, the following parameters were used: maximum gap to close was two frames, minimum length of TrackSegments from First Step was three frames, and segments were split. Cost functions and Kalman filter functions were set to “Microtubule plus-end dynamics.” U-track detected and traced EB3 comets automatically. To analyze the comet tracks further, the Python package “neuriteMT” was developed (<https://github.com/maxschelski/neuritemt>). This package uses all comet tracks from all time points to construct an image of the neuron. The image of the neuron was further processed using the pyNeurite package (described above) to obtain the skeletons of all neurites and the sorted points of the skeletons. The orientation of microtubules was obtained from the orientation of comet movement, relative to the orientation of neurites. For the first and last point of each comet, the closest neurite point was determined. If the last of the closest neurite points was further away or closer to the soma than the first of the closest neurite points, the comet was plus-end-out or minus-end-out, respectively. For comets where the first and last closest neurite points were the same, the direction of the comet over time was compared to the direction of the neurite skeleton at the closest neurite point. For each comet point, the closest neurite point was determined. The neurite direction at these points was then calculated from two points before up to 2 pixels after that neurite point. Comet direction was calculated at each time point, from the current time point to two time points further. The angle difference between the neurite direction and comet direction was calculated. If the angle difference was less than 70° or more than 110°, the comet at this time point was considered plus-end-out or minus-end-out, respectively. If the same orientation was determined for at least 65% of all time points, that orientation was used for the comet. If the same orientation was determined for less than 65% of all time points, no orientation was determined for the comet.

Analysis of spread and distribution of microtubule patches

All analysis was done with kymographs from manually traced neurites. For analyzing both the spread of photoactivated microtubule patches after LatB (fig. S4) and the distribution of photoconverted microtubule patches after recruiting the dynein motor to the plasma membrane (fig. S11), first, the background intensity in the neurite was determined. This was done by taking the lowest value from the first five columns (neurite pixels) in the first and the four last rows (time points) in the kymograph. For repeated photoconversions, the neurite background from later photoconversions always had to be at least as high as the highest neurite background in previous photoconversions. This was done because, with more photoconversions, the amount of photoconverted tubulin in the neuron also increased. For both photoactivation and for each interval of photoconversion, the neurite background was subtracted from the kymograph at the corresponding interval.

Analysis of the spread of photoactivated microtubule patches after LatA treatment (fig. S4) was done by removing nonzero pixels

from the edge of the kymograph at each time point after subtracting the neurite background from the kymograph. This was done by removing the pixel at the edge (anterograde or retrograde) with the lowest value until at least 10% of the summed intensity was removed. The distance between the most anterograde and retrograde pixels of the region—including the remaining 90% of the summed intensity—was the spread. To analyze the speed of anterograde and retrograde spread, the distance of the most anterograde and the most retrograde pixel in the spread from 1.1 μm retrograde and 1.1 μm anterograde of the start point of photoactivation was measured. The start point of photoactivation was determined for the first row (time point) as the pixel with the highest intensity after smoothing with a window size of 5 pixels. To obtain the speed of the spread, the row (time point) before the first with a signal lower than 1.3-fold above the neurite background was analyzed. The rapid dissipation of the photoactivated signal after LatA treatment compared to EtOH treatment led to an earlier time point being measured for LatA compared to EtOH-treated neurons.

The distribution of photoconverted microtubule patches after recruiting the dynein motor domain to the plasma membrane (fig. S11) was done by first determining the start point of the photoconversion. Without LatB treatment, the start point was determined as the pixel with the highest intensity at the first row (time point) after smoothing the intensity with a window size of 5 pixels; with LatB, the start point was directly extracted from the photoconverted regions defined during the experiment at the microscope. From that start point, the summed microtubule density (microtubule mass) after neurite background subtraction in all pixels more than 1.1 μm retrograde of the start point (retrograde), more than 1.1 μm anterograde of the start point (anterograde), or between 1.1 μm anterograde and 1.1 μm retrograde of the start point (stationary) was measured. The fraction of microtubule mass was calculated by dividing the microtubule mass in the respective region by that of the entire neurite. Only those rows (time points) up to one before the first row, with a signal of 1.8-fold (without LatB) or 1.3-fold (with LatB) above the neurite background, were analyzed. For experiments with LatB-treated neurons, the distribution was averaged from 3 to 4.5 min after dimerization. For experiments with neurons not treated with LatB, kymograph rows (time points) before speedup and up to 15 min after MT-RF speedup were analyzed. The time point of MT-RF speedup was determined manually from kymographs of CAMSAP3 along neurites.

Analysis of proximal and distal microtubule density

The proximal and distal microtubule density after recruiting the dynein motor domain to the plasma membrane in neurons treated with LatB (fig. S14) was analyzed using kymographs of the not-photoconverted tubulin. First, the neurite was automatically thresholded (see the “Automated analysis of neurite length from kymographs” section). Within the thresholded neurite, the microtubule densities in the first 5 μm (proximal) and within 5 to 10 μm from the neurite tip (distal) were analyzed for each time point. Proximal and distal microtubule densities were normalized to the average density in the first 22.5 min and then averaged before dimerization and 30 to 60 min after dimerization.

Analysis of KIF motor soma fraction after membrane recruitment

Soma fraction after recruiting KIF motor domains to the plasma membrane was done using ImageJ to manually create regions around the soma and the entire neuron. Images were thresholded, and the

average background-subtracted intensity was multiplied with the number of pixels in the thresholded area to obtain the total fluorescence in that area for each time point. The total fluorescence in the soma region was divided by the total fluorescence in the entire neuron to obtain the soma fraction of KIF motor domains. The soma fraction was averaged over a time range of 60 to 90 min after dimerization started.

Analysis of MT-RF from CAMSAP3 in dimerization experiments

For recruiting endogenous dynein and dynein motor domain compared to mutated dynein motor domain to the membrane (Fig. 9, C, D, F and G), average MT-RF was calculated before the addition of the dimerizer and 6 min to 1 hour after addition from CAMSAP3 traces. Cells with high bicDN expression levels or very low expression levels of membrane anchor CAAX were excluded from the analysis. For figs. S13 and S14B, MT-RF was averaged before dimerization and during the time from 3 to 30 min (fig. S13) or 30 to 45 min (fig. S14B) after dimerization. For fig. S16 (C to F), MT-RF was averaged before dimerization and at several time intervals after dimerization in relation to the time point when the soma fraction of KIF1a reached at least 25% for the first time (fig. S16, C and D) or the soma fraction of KIF13a reached at least 50% for the first time (fig. S16, E and F). Specifically, MT-RF was averaged after dimerization for the time more than 7 min before soma fraction increase (longer before), from 20 s to 7 min before soma fraction increase (right before), from 0 to 7 min after soma fraction increase (right after), and from 8 to 14 min after soma fraction increase (longer after). To check the effect of MT-RF speedup after recruiting dynein motor domain to the membrane (Fig. 10, B to F), MT-RF was averaged from 1.5 to 15 min after dimerization using CAMSAP3 traces. In neurons where dynein motor domain was recruited to the membrane, only neurites with MT-RF faster than 1 $\mu\text{m}/\text{min}$ were analyzed, while for the control, all neurites were analyzed independent of MT-RF speed.

Analysis of MT density and neurite length for dimerization experiments

MT density and neurite length were automatically extracted from kymographs of the tubulin channel (Fig. 10, C and D, and figs. S12C, S14F, and S16G). For Fig. 10C, immediate loss of MT density was obtained from the difference in normalized MT intensity from before dimerization to 4.5 to 9 min after dimerization in each neurite. For fig. S12C, the first microtubule density from the not-photoconverted tubulin channel was normalized to the average density in the first 4 min (six time frames). For fig. S14F, the growth rate was averaged from before dimerization and for the time from 30 to 60 min after dimerization. Then, microtubule density was measured in the last time frame of the video, which was 1 to 5 hours after dimerization started. For fig. S16G, retraction was calculated by comparing the neurite length to the average neurite length of 15 min before dimerization. Retraction was averaged for the time from 60 to 90 min after dimerization. Immediate retraction (Fig. 10D) was calculated by taking the difference in length before dimerization to the shortest length of 4.5 to 15 min after dimerization in each neurite. In the analysis for each neuron, values of all analyzed neurites were averaged.

Python scripts

All scripts were written using Python 3.7 or 3.8, with environments build with conda (Anaconda Inc., 2020; available from <https://docs.anaconda.com/>) and using the following packages: SciPy 1.6.2 (for FigureFlow) or 1.5.2 (<https://pypi.org/project/scipy/>) (67), sci-kit

image 0.17.2 or 0.18.1 (<https://pypi.org/project/scikit-image/>) (68), numpy 1.20.3 (<https://pypi.org/project/numpy/>) (69), seaborn 0.11.1 (for FigureFlow) or 0.11.0 (<https://pypi.org/project/seaborn/>) (70), scikit-posthocs 0.6.7 (<https://pypi.org/project/scikit-posthocs/>) (71), matplotlib 3.3.4 or 3.4.2 (<https://pypi.org/project/matplotlib/>) (72), pandas 1.3.0 (for FigureFlow) or 1.1.3 (<https://pandas.pydata.org/>) (73), and python-pptx 0.6.19 (<https://pypi.org/project/python-pptx/>).

Figure and movie preparation (FigureFlow)

We developed a Python package (FigureFlow) to prepare all figures and movies. The Python package allows for a highly standardized generation of publication-grade figures and movies using simple Python scripts. The package will be published as a separate protocol and will be made installable via pip. The package is already available via GitHub (<https://github.com/maxschelski/figureflow>). The features include simple data plotting (e.g., automatic data plotting, calculation, and annotation of statistical data in plots), simple display of representative cells (e.g., automatic calculation of cells closest to the mean, image extraction from ImageJ Hyperstack, definition of zoom regions, and annotation of channels or time points), and automated generation of movies with the same scripts and same flexibility as figure panel generation with movie-specific features (e.g., title pages, showing specific frames longer, and repeating movie). All panels are aligned accurately according to a user-defined grid. Illustrations were made in Adobe Illustrator and Microsoft PowerPoint, while the illustration of the 3D matrix in Fig. 2D (without neurons) is from BioRender (BioRender.com).

Statistics

For all data where more than two groups were compared, the non-parametric Kruskal-Wallis test (`scipy.stats.kruskal_wallis`) was first performed. If the resultant *P* value was below 0.05, all combinations of groups were compared. All statistical comparisons of the two groups were undertaken using the nonparametric Dunn's test (`scikit-posthocs.posthoc_dunn`), with Bonferroni correction. If only two groups of paired data were compared (Figs. 2, B and E, 4C, and 5B and figs. S3, S5, S6, S8, S14, B and D to F, S15, and S16, B and G), then the Wilcoxon signed-rank test for paired data (`scipy.stats.wilcoxon`) was performed.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.abo2336>

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Supplementary Materials for
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The PDF file includes:

Figs. S1 to S16
Legend for table S1
Table S2
Legends for movies S1 to S22

Other Supplementary Material for this manuscript includes the following:

Table S1
Movies S1 to S22

Supplementary Figures

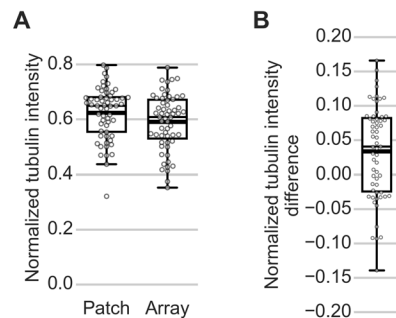


Fig. S1. Microtubules throughout the array are photoconverted.

Neurons expressing the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2, and the tubulin subtype TUBB5 fused to the halo-tag, stained with a far-red chemical fluorophore (JF646, Promega) were cultured for one day and then imaged. **(A and B)** After photoconversion of TUBB2a-mEos, z-stacks of the entirety of all neurites were acquired. Intensities were normalized to the slice with the highest intensity in that channel. The slices were analyzed from three z-planes further away from the glass to three z-planes closer to the glass around the highest intensity slices in both channels. The slices with the highest intensity (normalized to 1) in any channel were excluded from analysis. **(A)** The normalized intensity of each neurite was obtained as the average intensity across defined range of slices for the entire microtubule array as well as the photoconverted microtubule patch. Comparison of the normalized intensity for microtubule patch (photoconverted TUBB2a-mEos) and microtubule array (TUBB5-Halo stained with JF646) is shown. **(B)**. The difference of normalized intensity was calculated for each slice separately and then averaged across all slices for each microtubule patch. (55 neurites, 14 cells, N = 2 independent experiments). Thick line in boxplots represents the mean. p value > 0.05, Dunn's test.

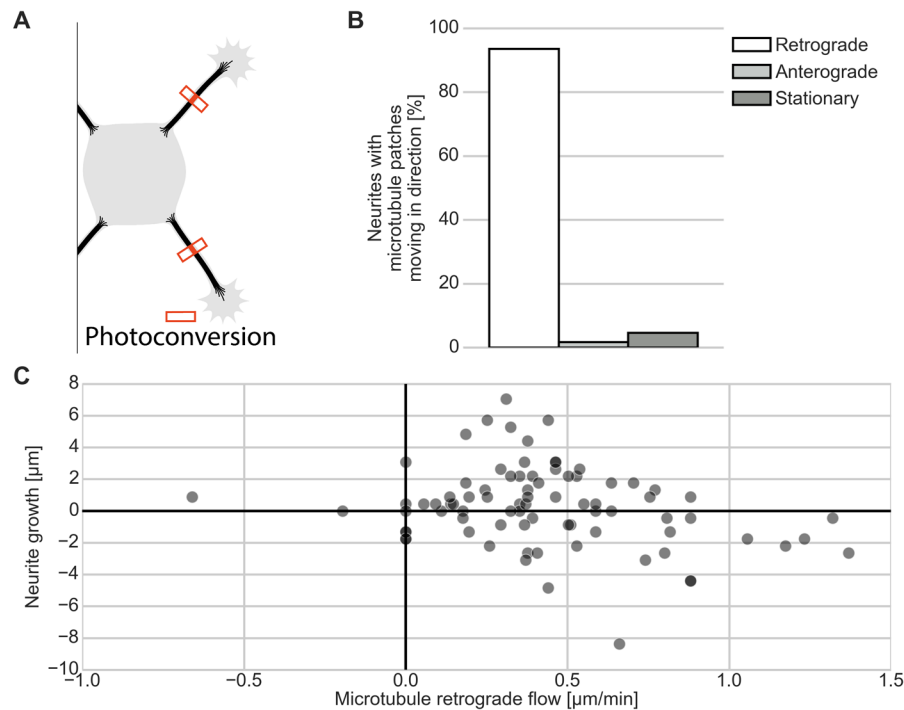


Fig. S2. Microtubules flow retrogradely in neurons in absence of neurite retraction.

Neurons expressing the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2 were cultured for one day and then imaged. (A) Illustration for photoconversion experiment in [B and C]. (B) Percentage of neurites with microtubules moving retrogradely (towards the soma), anterogradely (towards the neurite tip) and not moving from total neurites imaged. Data from Fig. 1B ($n = 71$ cells, $N = 11$ independent experiments). (C) Microtubule retrograde flow (x-axis) plotted against neurite growth during 10 min imaging intervals (y-axis). Part of image data from Fig. 1B was reanalyzed to measure neurite growth and microtubule retrograde flow together. ($n = 42$ cells, $N = 10$ independent experiments).

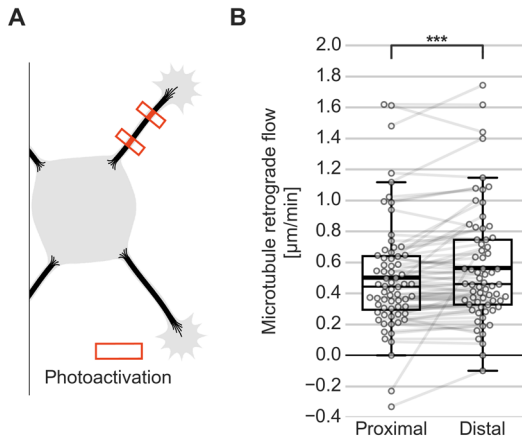


Fig. S3. MT-RF is similar in proximal and distal parts of the neurite.

Neurons expressing the tubulin subtype TUBB2a fused to the photoactivatable fluorophore Dronpa were imaged after one day in culture. **(A)** Illustration of the photoactivation experiment. **(B)** MT-RF measured for the distal and proximal photoactivated microtubule patches. Images used for analysis were the same as for [Fig. 1, E and F] ($n = 68$ cells, $N = 7$ independent experiments). Thick line in boxplots represents the mean. *** $P < 0.001$, Wilcoxon signed-rank test.

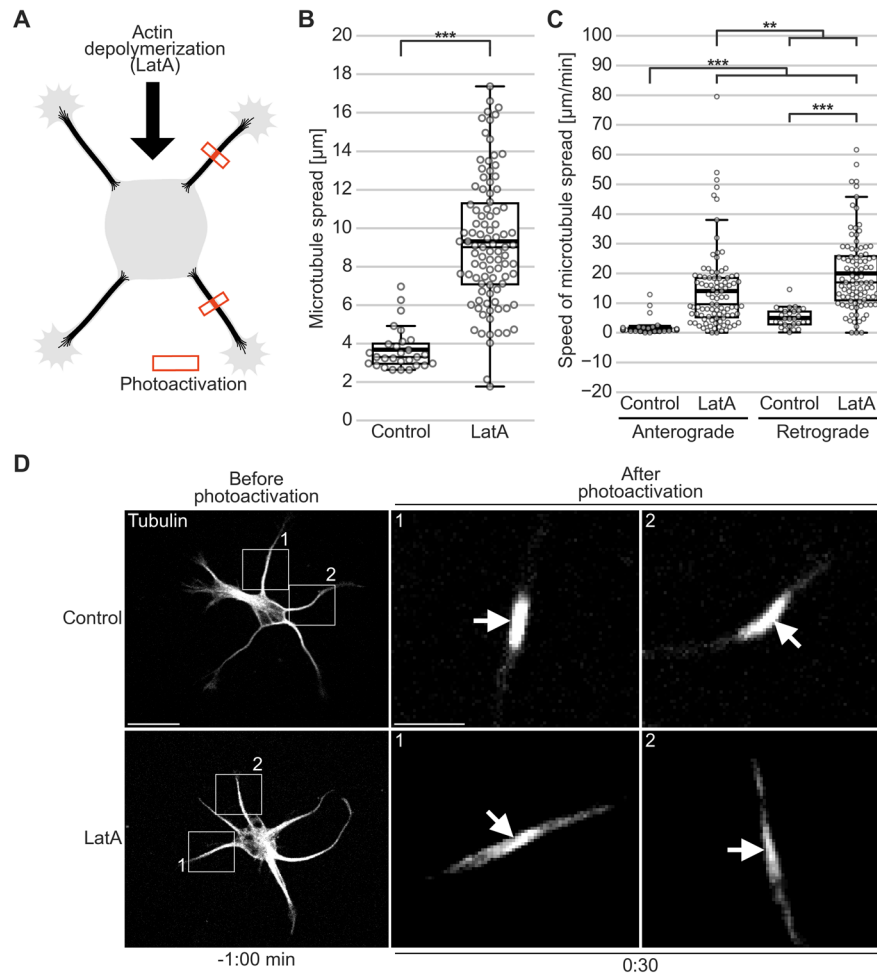


Fig. S4. After actin depolymerization microtubules move quickly in both directions.

Neurons expressing the tubulin subtype TUBB2a fused to the photoactivatable fluorophore Dronpa were cultured for one day, treated with $0.75 \mu\text{M}$ latrunculin A (LatA) for four hours and then microtubule patches were photoactivated and imaged for up to 10 minutes. **(A)** Illustration of experiment. **(B and C)** Spread of photoactivated tubulin (microtubule spread) was measured as the length around the point of photoactivation that includes 90% of photoactivated tubulin mass. **(B)** The length of the spread. **(C)** The distance of the furthest anterograde and retrograde point of the spread from $1.1 \mu\text{m}$ anterograde and retrograde of the photoactivation point was measured. This distance was divided by the time since photoactivation to obtain the speed of anterograde and retrograde spread. ($n = 28$ cells for control, $N=2$; $n=95$ cells for LatA, $N = 3$; N , number of independent experiments). **(D)** Representative neurons with photoactivation done at

0:00 min. Scale bar, 20 μm for overview images, 5 μm for zoomed images. Thick line in boxplots represents the mean. ** $P < 0.01$, *** $P < 0.001$, Wilcoxon signed-rank test for [B] and Kruskal Wallis multiple comparison with Dunn's posthoc test with Bonferroni correction for [C].

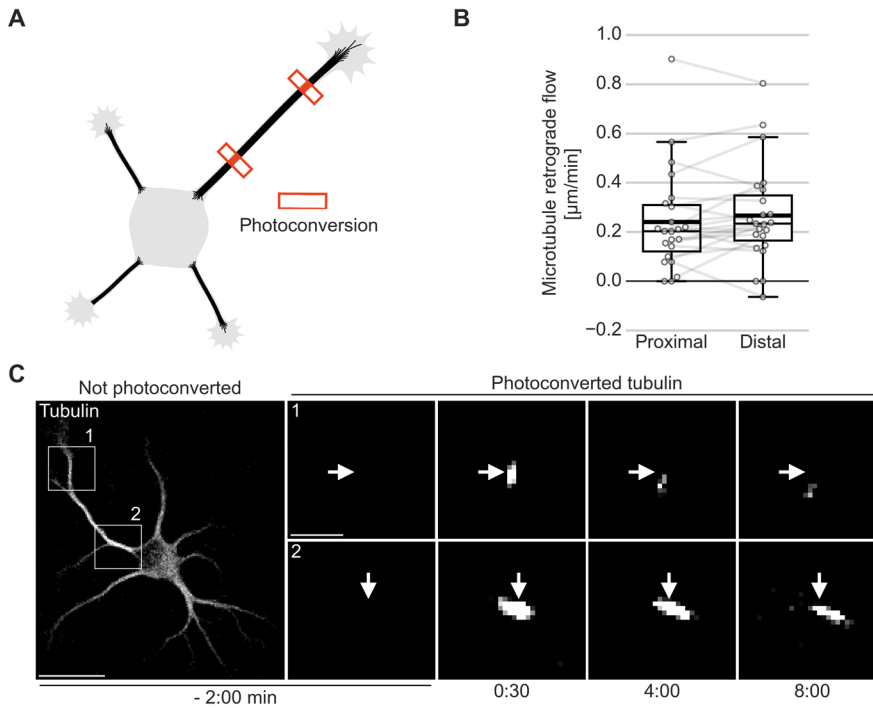


Fig. S5. MT-RF is similar in proximal and distal parts of the axon.

Neurons expressing the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2 were imaged after one day in culture. **(A)** Illustration of the photoconversion experiment. **(B)** MT-RF of the distal and proximal photoconverted microtubule patches. Only neurons with at least one microtubule patch moving were analyzed. ($n = 23$ cells, $N = 4$ independent experiments). Thick line in boxplots represents the mean. **(C)** Representative cell for [B]. Photoconversion was done at 0:00 min and white arrows point to the site of photoconversion. Scale bar, 20 μm for overview image, 5 μm for zoomed images. All p -value > 0.05 , Wilcoxon signed-rank test.

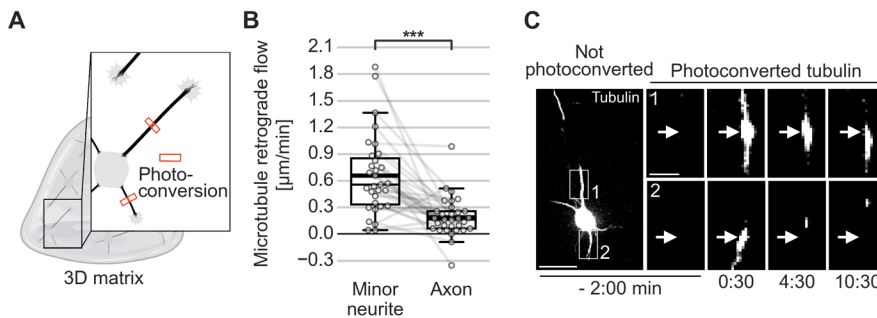


Fig. S6. In neurons grown in 3-dimensional matrices, MT-RF slows down in the axon.

The tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2, expressed in neurons and then imaged in neurons after one day in culture. **(A)** Illustration of photoconversion experiment for [B and C]. **(B)** MT-RF of neurons with axon grown in 3D. Patches that moved anterogradely were plotted with zero MT-RF. Thick line in boxplots shows mean. **(C)** Maximum intensity projection of representative cell from [B] with photoconversion done at 0:00 min and white arrows pointing to the site of photoconversion (n = 31 cells, N = 4 independent experiments). ***P < 0.001, Wilcoxon signed-rank test. Scale bar, 20 μm for overview image, 5 μm for zoomed images.

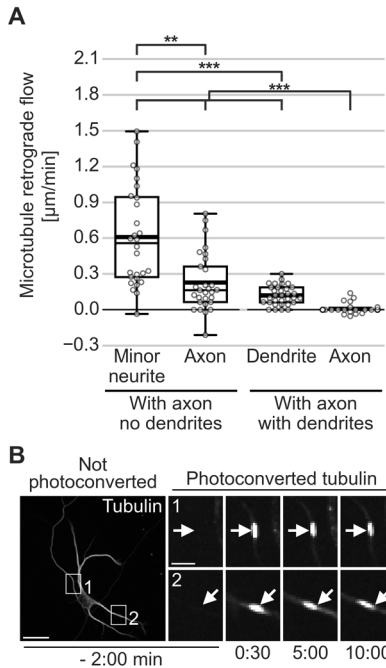


Fig. S7. Later in development MT-RF slows down in dendrites and axons.

The tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2, expressed in neurons and then imaged after one day (“With axon, no dendrites”) or after 6-to-7 days (“With axon, with dendrites”). **(A)** Quantification of MT-RF. Data from the condition “With axon, no dendrites” are the same as in [Fig. 2B] ($n = 31$ cells for neurons without dendrites, of those $n = 29$ cells for axons and $n = 28$ cells for minor neurites, $N = 8$ independent experiments; and $n = 32$ cells for neurons with dendrites, $N = 2$ independent experiments). Thick line in boxplots represents the mean. **(B)** Representative neuron with dendrites for [A] with photoconversion done at 0:00 min and white arrows pointing to the site of photoconversion. $**P < 0.01$, $***P < 0.001$, Kruskal Wallis multiple comparison with Dunn’s posthoc test with Bonferroni correction. Scale bar, $20 \mu\text{m}$ for overview image, $5 \mu\text{m}$ for zoomed images.

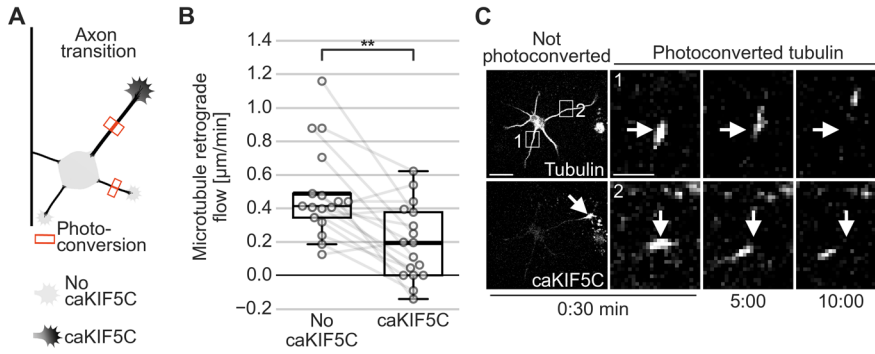


Fig. S8. MT-RF slows down in neurites with caKIF5C at the axon transition.

The tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2 and caKIF5C was fused to the fluorophore Cerulean3, expressed in neurons, and then imaged after one day. **(A)** Illustration of photoconversion experiments for [B and C]. **(B and C)** MT-RF in neurites without and with caKIF5C accumulation in neurons at the phase of axon growth. Neurons where the longest neurite was at least 30 μm long and at least 5 μm longer than the second -longest neurite were considered to be at the phase of axon growth ($n = 17$ cells, $N = 7$ independent experiments). White arrows in the photoconverted channel of [C] indicate areas of photoconversion and in the caKIF5C channel points to the growth cone with caKIF5C accumulation. Thick line in boxplots represents the mean. $**P < 0.01$, Wilcoxon signed-rank test. Scale bar, 20 μm for overview images, 5 μm for zoomed images.

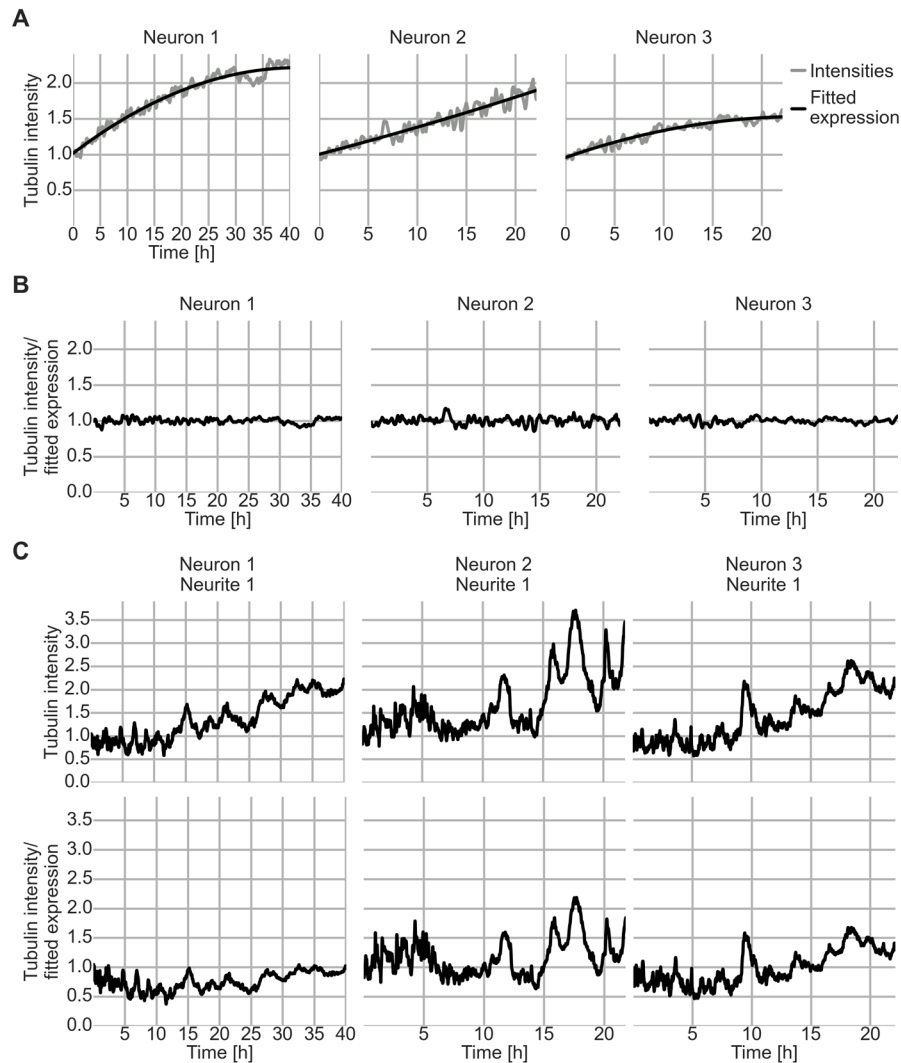


Fig. S9. Microtubule density increases due to increasing expression levels, which can be corrected for measuring density cycles.

Data shown is from dataset in [Fig. 5, B to E]. Neurons expressed the tubulin subtype TUBB2a fused to the fluorophore mScarlet and were imaged after one day in culture from before to after axon formation. (A) Average tubulin intensities in all neurites normalized to the first timeframe and the quadratic function fitted to the tubulin intensity over time normalized to the first timeframe. (B) Average tubulin intensities in all neurites divided by the fitted expression. (C) Average tubulin intensities and average tubulin intensities divided by the fitted expression for one neurite of each example neuron in [A and B].

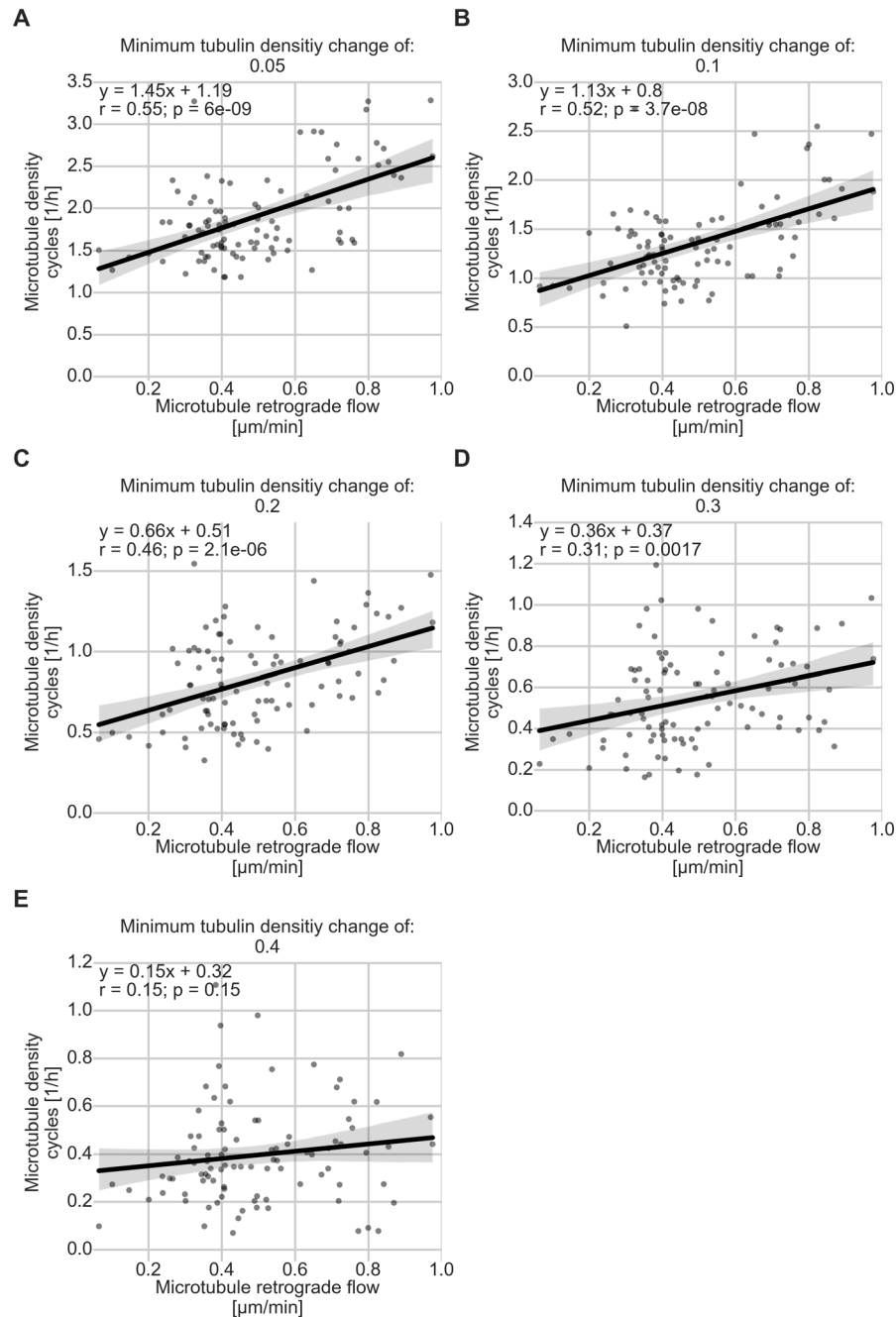


Fig. S10. The correlation of microtubule density cycles with MT-RF is robust.

Data from [Fig. 5E]. Neurons expressing the fluorophore mNeonGreen fused to CAMSAP3 and the tubulin subtype TUBB2a fused to the fluorophore mScarlet and were imaged after one day in culture. Microtubules and MT-RF were imaged simultaneously in neurons before axon formation. Microtubule density was calculated by dividing neuronal tubulin expression by average tubulin intensity, modelled as a quadratic function

of the average intensity in all neurites pooled for each timepoint. A change of microtubule density in a neurite of **(A)** 0.05, **(B)** 0.1, **(C)** 0.2, **(D)** 0.3 **(E)** 0.4 or more is half a cycle. An increase followed by a decrease, or vice versa, completes a full cycle.

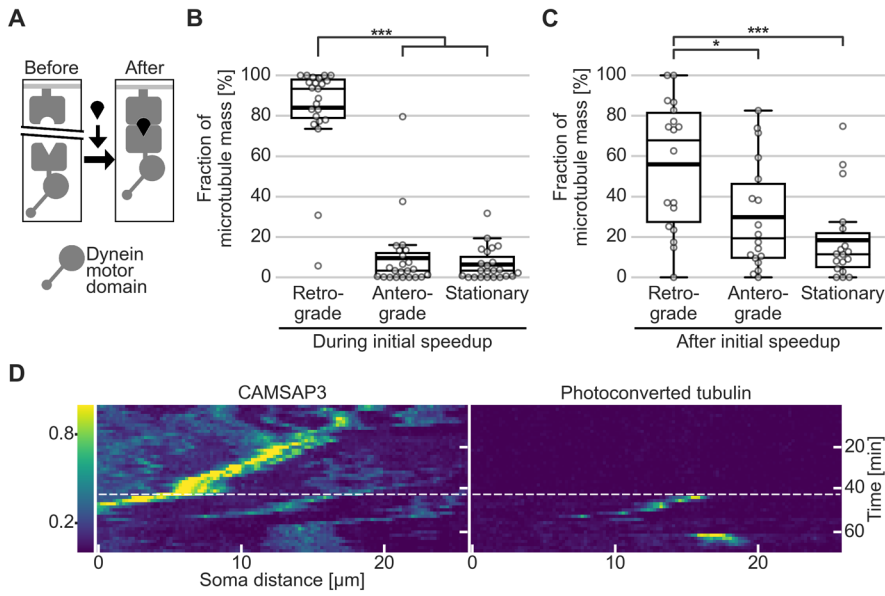


Fig. S11. Dynein motor domain recruitment to the membrane transiently moves microtubules retrogradely.

The dynein1 motor domain (dynein1 heavy chain 1 amino acids 1453 to 4644, Dync1h1motor) was recruited to the membrane anchor C2 with 62.5 nM of the rapalog A/C Dimerizer. Neurons expressed the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2, HaloTag fused to CAMSAP3, FRB-Dync1h1motor and FRB-C2-Cerulean3. HaloTag was stained with the fluorophore JaneliaFluor 646 to visualize CAMSAP3. The time of MT-RF speedup after dynein motor domain recruitment was determined manually from the CAMSAP3 signal. The distribution of photoconverted microtubules during speedup of MT-RF was measured. **(A)** Illustration of the dimerization experiment. **(B and C)** The distribution of photoconverted microtubule mass was measured **(B)** up to 10 min after speedup and **(C)** after 10 min. Photoconverted tubulin intensity more than 1.1 μm anterograde and more than 1.1 μm retrograde of the point with the highest intensity in the first frame was considered anterograde and retrograde, respectively. Intensity between these two points was considered stationary. (For [B] $n = 23$ cells, $N = 6$; for [C] $n=18$ cells, $N=5$; N , number of independent experiments) **(D)** Intensity of HaloTag-CAMSAP3 and photoconverted tubulin along the neurite (x-axis) over time (y-axis; kymograph). The left side is close to the soma. A trace from top right to lower left is MT-RF. The dashed line indicates the time right before MT-

RF speedup. Color-coded intensities shown (blue lower, yellow higher intensity). Thick line in boxplots represents the mean. * $P < 0.05$, *** $P < 0.001$, Kruskal Wallis multiple comparison with Dunn's posthoc test with Bonferroni correction.

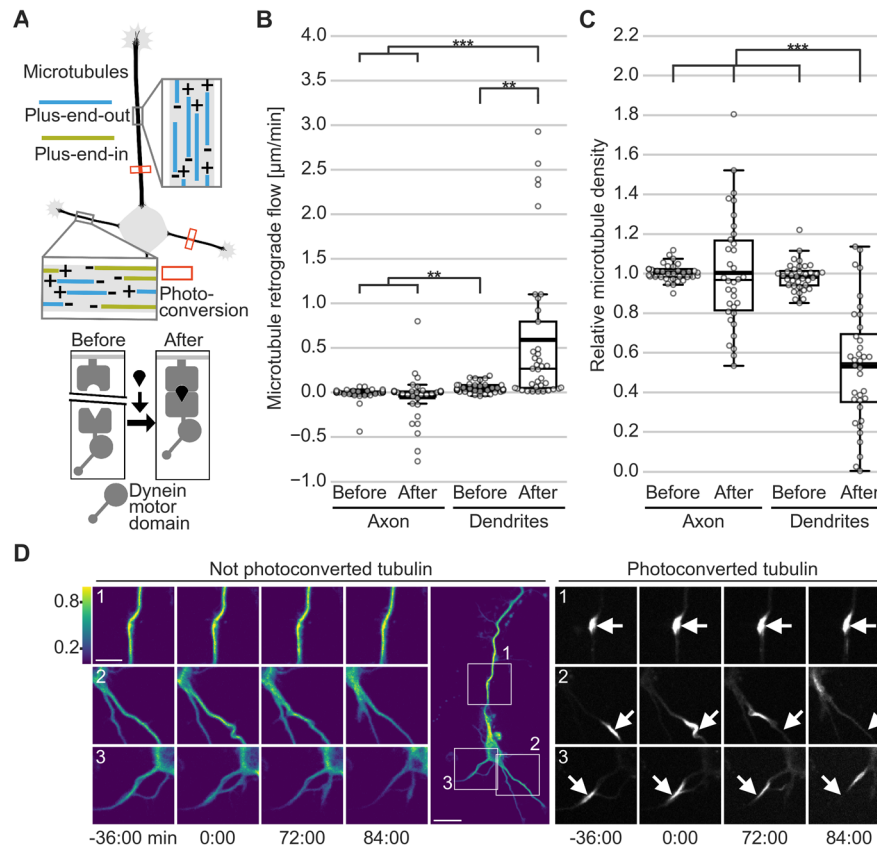


Fig. S12. Dynein motor domain recruitment to the membrane in old neurons speeds up MT-RF and leads to microtubule loss in dendrites but not in axons.

The dynein domain (dynein1 heavy chain 1 amino acids 1453 to 4644, Dync1h1motor) was recruited to the membrane anchor C2 after photoconversion of microtubule patches in neurons cultured for seven days. Neurons expressed the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2, FKBP-Halo-Dync1h1motor and FRB-C2-dCerulean3. Microtubule patches were photoconverted and neurons were imaged for 12 to 40 min, then microtubule patches were photoconverted again, 62.5 nM of the rapalog A/C dimerizer were added and neurons were imaged further for one hour to several hours. **(A)** Illustration of the dimerization experiment. **(B)** MT-RF was measured at the last timepoint before the end of the movie, before the microtubule patch entered the soma or before it dissolved. MT-RF was averaged for all dendrites of one neuron. (n = 36 cells for axon, n=37 cells for dendrite, N = 3 independent experiments) **(C)** Tubulin density from the not photoconverted channel was normalized to the average density in

the first 6 timeframes and then measured up to 5 h after dimerization started, at the last timeframe of the video (n = 34 cells for axon, n=36 cells for dendrite, N = 3 independent experiments) (**D**) Color coded tubulin density (blue lower, yellow higher intensity) and photoconverted tubulin of a representative cell with dimerization at 0:00 min. Scale bar, 20 μm for overview image, 5 μm for zoomed images. Thick line in boxplots represents the mean. **P < 0.01, ***P < 0.001, Kruskal Wallis multiple comparison with Dunn's posthoc test with Bonferroni correction.

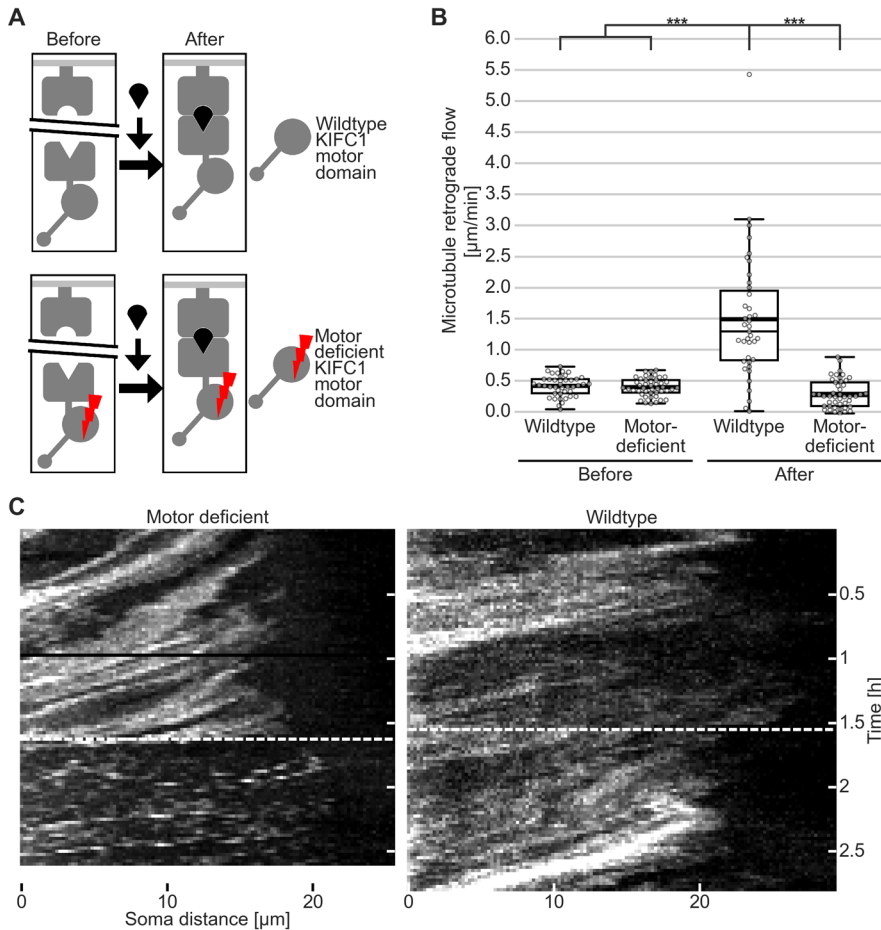


Fig. S13. Recruiting the minus-end directed motor KIFC1 to the plasma membrane speeds up MT-RF.

The wildtype motor domain of KIFC1 (amino acids 125-673) or a motor deficient KIFC1 motor domain (amino acids 125-673, N593K) was recruited to the membrane anchor C2. Neurons expressed the fluorophore mNeonGreen fused to CAMSAP3, FKBP-mScarlet-KIFC1 and FRB-C2- dCerulean3 and were imaged after one day in culture. **(A)** Illustration of the dimerization experiment. **(B)** Neurons were imaged for one to two hours, then 0.5 μM of the rapalog A/C dimerizer was added to start the recruitment. Imaging was continued for approximately one more hour. MT-RF was analyzed from 3 to 30 min after dimerization. (n = 45 cells for motor deficient KIFC1, n=42 cells for wildtype KIFC1 before and n=39 for wildtype KIFC1 after, N = 5 independent experiments) Thick line in boxplots represents the mean. **(C)** mNeonGreen-CAMSAP3 intensity along neurites (x-axis) over

time (y-axis; kymograph). The left side is closer to the soma and therefore a line from top right to bottom left shows MT-RF. The white dashes line indicates the timepoint of dimerization. *** $P < 0.001$, Kruskal Wallis multiple comparison with Dunn's posthoc test with Bonferroni correction.

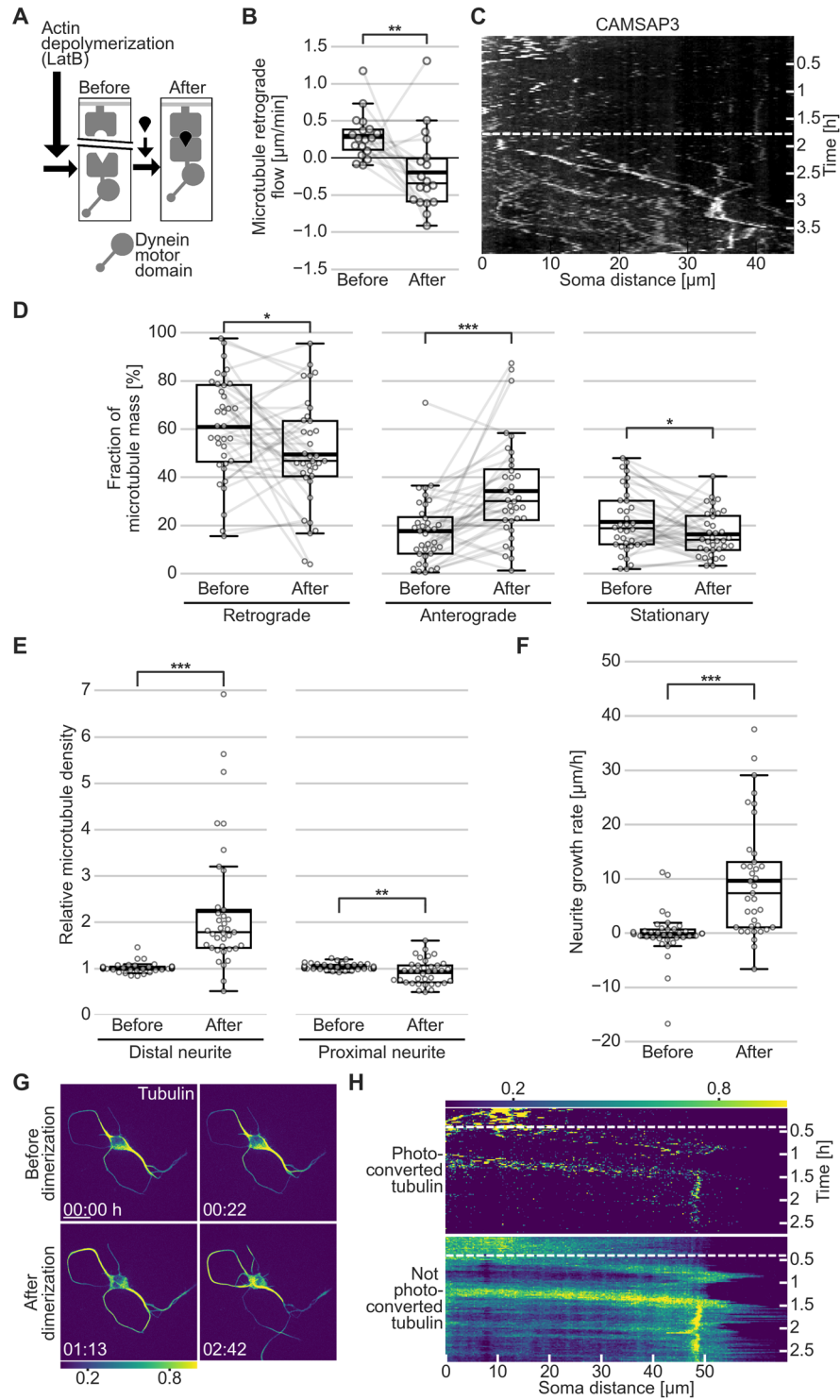


Fig. S14. Recruiting the dynein motor domain to the plasma membrane after actin depolymerization leads to anterograde microtubule flow and neurite growth.

The dynein domain (dynein1 heavy chain 1 amino acids 1453 to 4644, Dync1h1motor) was recruited to the membrane anchor C2 after 4 to 8 h treatment with 10 μ M latrunculin B. Neurons expressed FKBP-Halo-Dync1h1motor, FRB-C2-dCerulean3 and (B and C) the fluorophore mNeonGreen fused to CAMSAP3 or (D to H) the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2. Neurons were imaged after one day in culture (B and C) for 45 to 105 min or (D to H) 24 to 54 min, then 62.5 nM of the rapalog A/C dimerizer were added and imaging was continued. **(A)** Illustration of the dimerization experiment. **(B)** MT-RF was measured from CAMSAP3 traces before dimerization and from 30 to 60 min after dimerization. (n = 18 cells, N=2 independent experiments) **(C)** CAMSAP intensity along neurites (x-axis) over time (y-axis; kymograph). The dashed line indicates the time of dimerization. **(D to F)** Before and directly after dimerization, microtubule patches were photoconverted and imaged. **(D)** Photoconverted tubulin more than 1.1 μ m anterograde and more than 1.1 μ m retrograde of the photoconversion point was averaged from 3 to 4.5 min after photoconversion, before and after dimerization. The fraction of microtubule mass before and after dimerization were compared. **(E)** Tubulin density was measured in the proximal 5 μ m and the region of 5 to 10 μ m from the distal end of each neurite and normalized to the average density in the respective region in the first 22.5 min. Relative microtubule density was averaged before dimerization and from 30 to 60 min after dimerization. Relative microtubule density was compared before and after dimerization. **(F)** Neurite growth rate was averaged over all timepoints before dimerization and for 0 to 30 min after dimerization. (For [E and F] n = 37, of those n = 36 cells for [D]; N = 5 independent experiments) **(G)** Color coded tubulin density (blue lower, yellow higher intensity) of a representative cell before and after dimerization at 00:24 h. Scale bar, 20 μ m. **(H)** Color coded photoconverted and not photoconverted microtubule density (blue lower, yellow higher intensity) along neurite (x-axis) over time (y-axis; kymograph) for one neurite from [G]. The soma is close to the left side. A trace from top right to bottom left indicates retrograde movement while a trace from top left to bottom right indicates anterograde movement. The dashed white line indicates the time of

dimerization. Thick line in boxplots represents the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Wilcoxon signed-rank test.

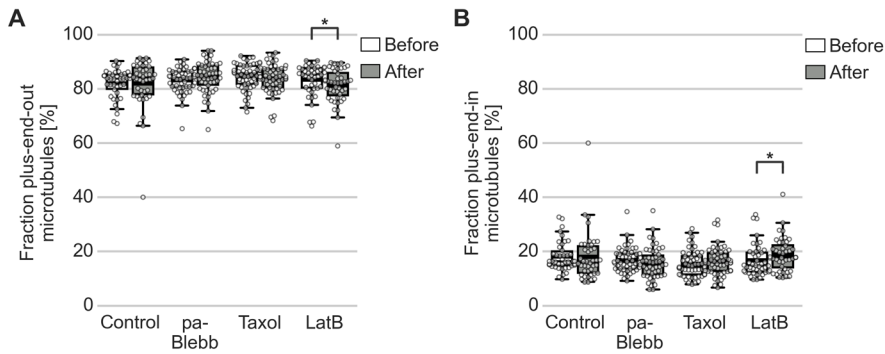


Fig. S15. Treatments that change MT-RF have little effect on MT orientation.

Neurons expressed the microtubule plus tip tracker EB3 fused to the fluorophore mNeonGreen. After one day in culture neurons were imaged for 1 min, then were treated with 6 nM taxol, 40 μ M para-amino Blebbistatin (pa-Blebb) or 10 μ M latrunculin B (LatB) for 3.5 to 7 hours and neurons were imaged again for 1 min. Microtubule orientation was measured from EB3 comet movement direction relative to the soma. EB3 comets were traced using the plusTipTracker function of u-track and traces automatically processed with a custom-made Python package. **(A and B)** Fraction of microtubules **(A)** plus-end-out and **(B)** plus-end-in. (n = 42 cells for control, n = 50 cells for pa-Blebb, n = 55 cells for taxol, n = 44 cells for LatB; N = 3 independent experiments). Thick line in boxplots represents the mean. *P < 0.05, Wilcoxon signed-rank test.

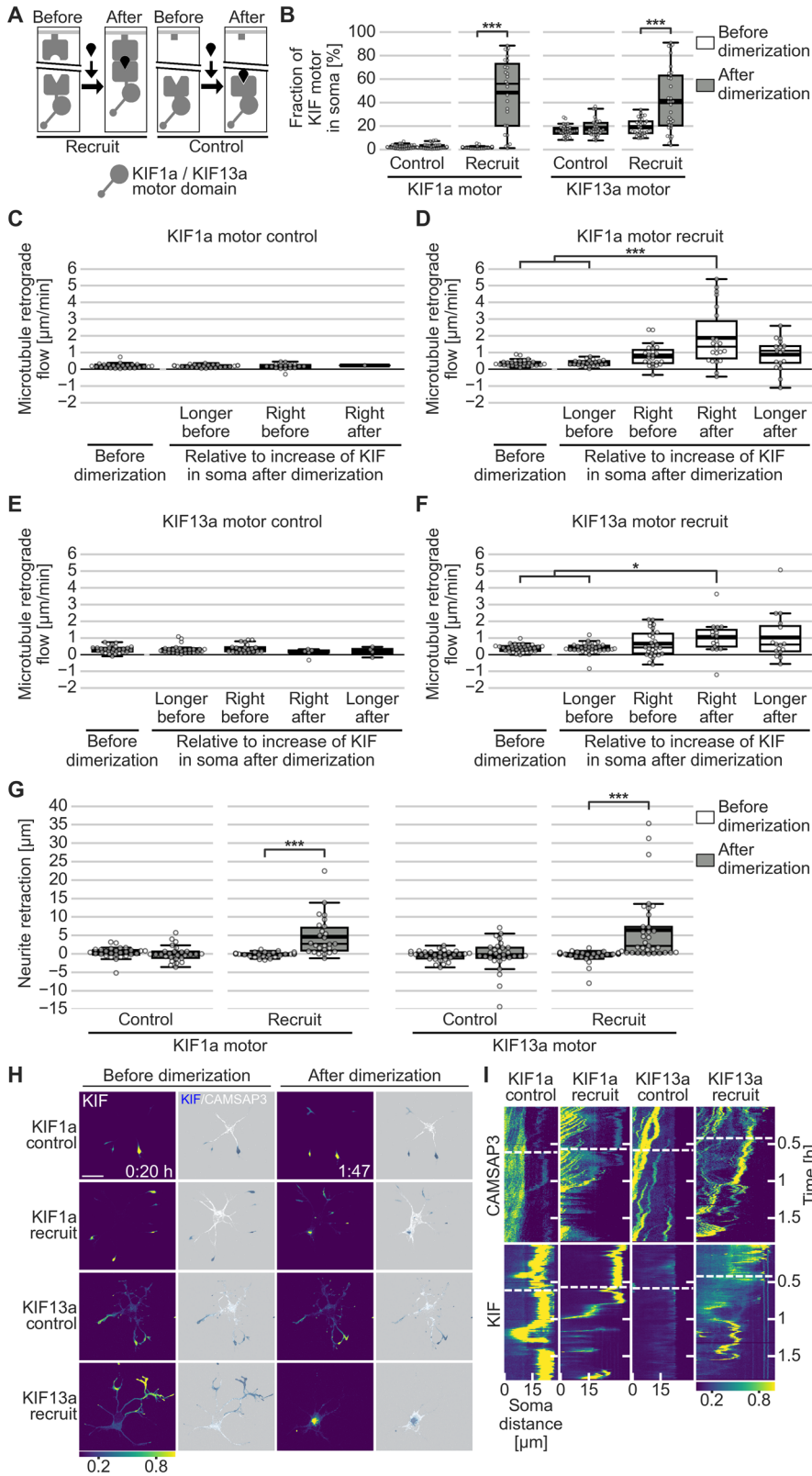


Fig. S16. Recruiting kinesin motor domains to the membrane speeds up MT-RF and retracts neurites after motor domain movement to the soma.

The motor domains of the kinesins KIF1a (amino acids 1-405) and KIF13a (amino acids 1-639) were recruited to the membrane anchor C2 using the rapalog system. Neurons expressed the fluorophore mNeonGreen fused to CAMSAP3, FRB-C2-Cerulean3 (recruit) or Cerulean3-C2-Cerulean3 (control) and KIF1aMotor-mScarlet-FKBP (KIF1a motor) or KIF13aMotor-mScarlet-FKBP (KIF13a motor). Neurons were imaged after one day in culture for 16 to 60 min, then 62.5 nM of the rapalog A/C dimerizer was added and neurons were imaged further. **(A)** Illustration of the dimerization experiment. **(B)** Fraction of KIF mass in the soma was calculated as the fraction from the KIF mass in the entire neuron. The soma fraction was averaged before dimerization and from 60 to 90 min after dimerization. Soma fraction were compared before and after dimerization. **(C to F)** MT-RF was measured from CAMSAP3 traces and averaged in time ranges relative to the timepoint of dimerization and the timepoint with **(C and D)** a soma fraction of KIF1a of at least 25% or **(E and F)** a soma fraction of KIF13a of at least 50%. MT-RF was averaged before dimerization and for several time ranges after dimerization: more than 7 min before soma fraction increase (longer before), from 0 to 7 min before soma fraction increase (right before), from 0 to 7 min after soma fraction increase (right after) and from 8 to 14 min after soma fraction increase (longer after). If a cell did not reach the defined threshold of soma fraction of the kinesin motor, “longer before” and “right before” were relative to the end of the movie and the cell did not have data for “right after” and “longer after”. For **[E]** no cell had data from “longer after” soma fraction increase. **(G)** Neurite retraction was calculated in relation to the average neurite length of the last 15 min before dimerization and averaged from 60 to 90 min after dimerization. Retraction was compared before and after dimerization. **(H)** Representative neurons showing KIF distribution in the neuron before and after dimerization. Color coded KIF intensity shown (blue lower, yellow higher intensity). Dimerization was done between 00:25 and 00:40 min. Scale bar, 20µm. **(I)** Color coded CAMSAP3 and KIF intensity (blue lower, yellow higher intensity) along one neurite from each neuron in **[H]** (x-axis) over time (y-axis; kymograph). (for KIF1a control n = 30 for **[B]**, of those n = 25 for **[C]** and n = 27 cells for **[G]**; for KIF1a recruit for **[B, D and G]** n = 27 cells; for KIF13a control for **[B]** n = 31 cells, of those n = 28 cells for **[E]** and

n = 30 cells for [G]; for KIF13a recruit n = 34 cells for [B], of those n = 33 cells for [F and G]). Thick line in boxplots represents the mean. ***P < 0.001, *P < 0.05, Wilcoxon signed-rank test for [B and G] and Kruskal Wallis multiple comparison with Dunn's posthoc test with Bonferroni correction for [C to F].

Supplementary Tables

Table S1. List of primers.

Related to methods, section DNA constructs. Provided as separate file.

Table S2. List of plasmids used for transfections.

Related to methods, section Neuron transfections.

Name	Source	Identifier
pBa-tdTomato-bicDN-FKBP	(60)	Addgene plasmid #64205
pHalo-N1-TUBB5-Halo	(13)	Addgene plasmid #64691
pBetaActin-tandem-mCherry	Sebastian Dupraz	N/A
pBetaActin-EB3-mNeonGreen	Stanislav Vinopal	N/A
pBetaActin-TUBB2a-mEos3.2	This paper	Addgene plasmid #191320
pBetaActin-TUBB2a-Dronpa	This paper	Addgene plasmid #191321
pBetaActin-mNeonGreen-CAMSAP3	This paper	Addgene plasmid #191322
pBetaActin-TUBB2a-mNeonGreen	This paper	Addgene plasmid #191323
pBetaActin-TUBB2a-mScarlet	This paper	Addgene plasmid #191324
pBetaActin-IC2N-dCer3	This paper	Addgene plasmid #191325
pBetaActin-FRB-dCer3-CAAX	This paper	Addgene plasmid #191326
pBetaActin-FRB-C2-dCer3	This paper	Addgene plasmid #191327
pBetaActin-dCer3-C2-dCer3	This paper	Addgene plasmid #191328
pBetaActin-Halo-CAMSAP3	This paper	Addgene plasmid #191329
pBetaActin-FKBP-mScarlet-Dync1h1motor	This paper	Addgene plasmid #191330
pBetaActin-FKBP-mScarlet-Dync1h1motorK2599T	This paper	Addgene plasmid #191331
pBetaActin-FKBP-Dync1h1motor	This paper	Addgene plasmid #191332
pBetaActin-FKBP-Halo-Dync1h1motor	This paper	Addgene plasmid #191333
pBetaActin-FKBP-mScarlet-KIFC1motor	This paper	Addgene plasmid #191334
pBetaActin-FKBP-mScarlet-KIFC1motorN593K	This paper	Addgene plasmid #191335
pBetaActin-KIF1aMotor-mScarlet-FKBP	This paper	Addgene plasmid #191336
pBetaActin-KIF13aMotor-mScarlet-FKBP	This paper	Addgene plasmid #191337
pBetaActin-TUBB2a-mEos3.2-mEos3.2-mEos3.2	This paper	Addgene plasmid #191338

Supplementary Movies

Movie S1. Microtubules move retrogradely towards the soma.

Data from Fig. 1C. Neurons expressing tubulin subtype TUBB2a fused to photoconvertible fluorophore mEos3.2 were cultured for one day and then imaged before axon development. The filled white circle indicates the time of photoconversion and photoactivation at 0:00 min, with white arrows indicating the site of photoconversion. Scale bar, 20 μm .

Movie S2. Microtubules move retrogradely in a synchronous manner.

Data from Fig. 1F. Neurons without an axon expressed the tubulin subtype TUBB2a fused to the photoactivatable fluorophore Dronpa, were cultured for one day and then imaged. The filled white circle indicates the time of photoconversion and photoactivation at 0:00 min, with white arrows indicating the sites of photoconversion. Scale bar, 20 μm .

Movie S3. MT-RF slows down in the axon.

Data from Fig. 2C. The tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2, expressed in neurons imaged after one day in culture. The filled white circle indicates the time of photoconversion at 0:00 min with white arrows indicating the sites of photoconversion. Scale bar, 20 μm .

Movie S4. MT-RF is similar in proximal and distal parts of the axon.

Data from fig. S5C. Neurons expressing the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2 were imaged after one day in culture. The filled white circle indicates the time of photoconversion at 0:00 min, while white arrows indicate the sites of photoconversion. Scale bar, 20 μ m.

Movie S5. MT-RF slows down in axons of neurons cultured in 3D.

Data from Fig. S6C. Neurons expressing tubulin subtype TUBB2a fused to photoconvertible fluorophore mEos3.2 were cultured in 3D collagen matrices and imaged after one day in culture. Maximum intensity projection is shown. The filled white circle indicates the time of photoconversion at 0:00 min with white arrows indicating the sites of photoconversion. Scale bar, 20 μ m.

Movie S6. MT-RF slows down in axons of neurons in brain slices.

Data from Fig. 2F. To image neurons in brain slices, embryonic cortices were electroporated *ex utero* with TUBB2a fused to triple mEos3.2, brains were sliced coronally, kept in culture for 2 days and then imaged. Maximum intensity projection is shown. The filled white circle indicates the time of photoconversion at 0:00 min with white arrows indicating the sites of photoconversion. Scale bar, 20 μ m.

Movie S7. Later in development MT-RF slows down in dendrites and axons.

Data from fig. S7B. The tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2, expressed in neurons and then imaged after 6 to 7 days. The filled white circle indicates the time of photoconversion at 0:00 min with white arrows indicating the sites of photoconversion. Scale bar, 20 μ m.

Movie S8. MT-RF slows down shortly after axon outgrowth.

Data from Fig. 3, D and E. The microtubule minus-end binding protein CAMSAP3 was fused to the fluorophore mNeonGreen and expressed together with the cytosolic marker td-mCherry (cytosol). Fusion proteins were expressed in neurons and imaged after one day in culture. The white arrows indicate the timepoint at which the axon grew out. Scale bar, 20 μm .

Movie S9. MT-RF does not continuously slow down before axon formation in neurites with axon-like properties.

Data from Fig. 4D. The marker for axon-like properties caKIF5C was fused to the fluorophore Cerulean3 and expressed together with the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2. Neurons without an axon were imaged after one day in culture. White arrows in the photoconverted and non-photoconverted channels indicate the site that was photoconverted at time 0:00. The white arrow in the caKIF5C channel points to neurites with caKIF5C accumulation. Scale bar, 20 μm .

Movie S10. Microtubule density cycles are reduced in the axon.

Data from Fig. 5, C and D. The tubulin subtype TUBB2a was fused to the fluorophore mScarlet and expressed in neurons. Neurons were imaged after one day in culture. The bigger white arrow appears at the time when the axon grows out and points to the neurite that becomes the axon. Tubulin intensity for the two neurites marked with smaller white arrows was calculated by normalizing the average tubulin intensity in the neurite by the tubulin expression level in the neuron, modelled as a linear function of the average intensity in all neurites for each timepoint. Scale bar, 20 μm .

Movie S11. Treatment with compounds that stabilize multiple axon identities slows down MT-RF.

Data from Fig. 6C. The tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2 and expressed in neurons. Neurons were cultured for one day and then treated with control (DMSO), 40 μ M pa-Blebb or 6 nM taxol for 20 – 240 min and directly imaged. The filled white circle indicates the time of photoconversion at 0:00 min with white arrows indicating the site of photoconversion. Scale bar, 20 μ m.

Movie S12. Treatment with pa-Blebb and taxol reduces microtubule density cycles.

Data from Fig. 7, C and D. The tubulin subtype TUBB2a was fused to the fluorophore mNeonGreen and expressed in neurons. After one day in culture, neurons were treated with control (DMSO), 40 μ M pa-Blebb or 6 nM taxol for 120 min and then imaged. Tubulin intensity was obtained by normalizing the average tubulin intensity in the neurite (indicated by white arrows) by the tubulin expression level in the neuron, modelled as a linear function of the average intensity in all neurites for each timepoint. Scale bar, 20 μ m.

Movie S13. Inhibiting dynein slows down MT-RF.

Data from Fig. 8, D and E. Tubulin subtype TUBB2a was fused to the photoconvertible fluorophore mEos3.2. for chronically inhibiting dynein, I2CN-Cerulean3 was expressed in neurons. Neurons were imaged after one day in culture. For acutely inhibiting dynein, neurons were treated with control (DMSO) or 50 μ M Ciliobrevin-A for 30 min directly before imaging. The filled white circle indicates the time of photoconversion with white arrows indicating the site of photoconversion. Scale bar, 20 μ m.

Movie S14. Recruiting endogenous dynein to the membrane speeds up MT-RF.

Data from Fig. 9D. mNeonGreen-CAMSAP3, tdTomato-bicDN-FKBP and FRB-Cerulean3-CAAX were expressed in neurons. After one day in culture, neurons were

imaged for 24 min, at 0:00 min the 0.5 μ M of the rapalog A/C Dimerizer was added, as indicated by the filled white circle, and imaging continued for one hour. Scale bar, 20 μ m.

Movie S15. Dynein motor domain recruitment to the membrane in old neurons speeds up MT-RF and leads to microtubule loss in dendrites but not in axons.

Data from fig. S12D. The dynein domain (dynein1 heavy chain 1 amino acids 1453 to 4644, Dync1h1motor) was recruited to the membrane anchor C2 after photoconversion of microtubule patches in neurons cultured for seven days. Neurons expressed the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2, FKBP-Halo-Dync1h1motor and FRB-C2-dCerulean3. Microtubule patches were photoconverted and neurons were imaged, then microtubule patches were photoconverted again, 62.5 nM of the rapalog A/C dimerizer were added and neurons were imaged further. The filled white circle indicates the time at which the dimerizer was added at 0:00 min with white arrows indicating the site of photoconversion. Scale bar, 20 μ m.

Movie S16. Recruiting dynein motor domain to the membrane speeds up MT-RF but not recruiting motor deficient dynein motor domain.

Data from neurons shown in Fig. 9G. mNeonGreen-CAMSAP3, FRB-C2-Cerulean3 and FKBP-mScarlet-Dync1h1motor (wildtype) or FKBP-mScarlet-Dync1h1motorK2599T (motor-deficient) were expressed in neurons. After one day in culture, neurons were imaged for around one hour, then at 0:00 min, indicated by the filled white circle, 0.5 μ M of the rapalog A/C Dimerizer was added, and imaging continued for one hour. Scale bar, 20 μ m.

Movie S17. Recruiting the minus-end directed motor KIFC1 to the membrane speeds up MT-RF.

Data from neuron shown in fig. S13C. The wildtype motor domain of KIFC1 (amino acids 125-673) or a motor deficient KIFC1 motor domain (amino acids 125-673, N593K) was

recruited to the membrane anchor C2. Neurons expressed the fluorophore mNeonGreen fused to CAMSAP3, FKBP-mScarlet-KIFC1 and FRB-C2- dCerulean3 and were imaged after one day in culture. Neurons were imaged for one to two hours, then 0.5 μ M of the rapalog A/C dimerizer was added to start the recruitment. The filled white circle indicates the time at which the dimerizer was added at 0:00 min. Scale bar, 20 μ m.

Movie S18. Recruiting dynein motor domain to the membrane enhances immediate loss of MT density, increases MT density cycles and induces retraction.

Data from Fig. 10, E and F. Neurons expressed mNeonGreen-CAMSAP3, FKBP-mScarlet-Dync1h1 and FRB-C2-Cerulean3 (speedup) or C2-Cerulean3 (control) and were imaged after one day in culture. After imaging for around 30 min, at 0:00 min 62.5 nM of the rapalog A/C Dimerizer was added, and imaging continued for another 90 min. The filled white circle indicates the time at which the dimerizer was added at 0:00 min. White arrows indicate neurites that retracted shortly after dimerization. Scale bar, 20 μ m.

Movie S19. Recruiting the dynein motor domain to the membrane after actin depolymerization increases anterograde microtubule flow.

Data from neuron shown in fig. S14C. The dynein domain (dynein1 heavy chain 1 amino acids 1453 to 4644, Dync1h1motor) was recruited to the membrane anchor C2 after 4 to 8 h treatment with 10 μ M latrunculin B. Neurons expressed FKBP-Halo-Dync1h1motor, FRB-C2-dCerulean3 and the fluorophore mNeonGreen fused to CAMSAP3. Neurons were imaged after one day in culture, then 62.5 nM of the rapalog A/C dimerizer was added and imaging was continued. The filled white circle indicates the time at which the dimerizer was added at 0:00 min. Scale bar, 20 μ m.

Movie S20. Recruiting the dynein motor domain to the membrane after actin depolymerization increases anterograde microtubule flow and neurite growth.

Data from neurons shown in fig. S14, G and H. The dynein domain (dynein1 heavy chain 1 amino acids 1453 to 4644, Dync1h1motor) was recruited to the membrane anchor C2 after 4 to 8 h treatment with 10 μ M latrunculin B. Neurons expressed FKBP-Halo-Dync1h1motor, FRB-C2-dCerulean3 and the tubulin subtype TUBB2a fused to the photoconvertible fluorophore mEos3.2. Neurons were imaged after one day in culture, then 62.5 nM of the rapalog A/C dimerizer were added and imaging was continued. The filled white circle indicates the time at which the dimerizer was added at 0:00 min while white arrows indicate the sites of photoconversion. Scale bar, 20 μ m.

Movie S21. Treatments that change MT-RF have little effect on MT orientation.

Data from representative neurons of data in fig. S15. Neurons expressed the microtubule plus tip tracker EB3 fused to the fluorophore mNeonGreen. After one day in culture neurons were imaged for 1 min, then were treated with 6 nM taxol, 40 μ M para-amino Blebbistatin (pa-Blebb) or 10 μ M latrunculin B (LatB) for 3.5 to 7 hours and neurons were imaged again for 1 min. The filled white circle indicates the time after treatment. Scale bar, 20 μ m.

Movie S22. Recruiting kinesin motor domains to the membrane speeds up MT-RF and retracts neurites after motor domain movement to the soma.

Data from neurons shown in fig. S16, H and I. The motor domains of the kinesins KIF1a (amino acids 1-405) and KIF13a (amino acids 1-639) were recruited to the membrane anchor C2 using the rapalog system. Neurons expressed the fluorophore mNeonGreen fused to CAMSAP3, FRB-C2-Cerulean3 (recruit) or Cerulean3-C2-Cerulean3 (control) and KIF1aMotor-mScarlet-FKBP (KIF1a motor) or KIF13aMotor-mScarlet-FKBP (KIF13a motor). Neurons were imaged after one day in culture for 16 to 60 min, then 62.5 nM of the rapalog A/C dimerizer were added and neurons were imaged further. The filled white circle indicates the time at which the dimerizer was added at 0:00 min. Scale bar, 20 μ m.

Appendix 2: Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance

Title: Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance

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Abstract:

Distinct microtubule arrays form in axons and dendrites defining their functions in neurons. These arrays are thought to develop through separate processes. Here we challenge this view by showing that axons and dendrites develop by a unifying process: slowdown of microtubule retrograde flow (MT-RF), a recently discovered mechanism of microtubule dynamics. By integrating quantitative data of microtubule dynamics and distributions into a newly developed biophysical model of microtubules across developmental stages, we uncover that MT-RF interacts with microtubule stability and nucleation. Without MT-RF slowdown, this interaction suppresses development and maintenance of axonal and dendritic microtubule arrays. In axons, MT-RF slowdown enables microtubules to reach the tip, even at long distances, supporting axon growth. In dendrites, late MT-RF slowdown facilitates an efficient increase in stable microtubules. Thus, our data-integrated model reveals MT-RF slowdown as central for the development of distinct neuronal compartments, providing a unifying mechanism for microtubule-related physiology.

Main:

Axons and dendrites differ in their morphology and molecular composition; this polarization enables neurons to function^{1,2}. Differences between axons and dendrites are thought to be established through separate developmental processes². Developing neurites, axons, and dendrites differ in their microtubule (MT) array^{3,4}. In developing neurites, the majority of MTs orient with their growing plus-end towards the neurite tip (plus-end-out), and a smaller fraction of MTs orients towards the soma (plus-end-in)⁵. In axons, MTs form a uniformly oriented array with plus-end-out MTs⁵⁻⁷, while the MT array in dendrites consists of MTs with both orientations in equal parts^{5,6}. These MT differences direct polarized transport into axons and dendrites^{8,9}. This has led to a large body of research addressing which separate mechanisms in either axons or dendrites enable such a distinct organization^{3,8-11}.

Here, we asked instead whether the axonal and dendritic MT arrays could be created through one central MT regulatory mechanism. We hypothesized that such a central mechanism could provide specific and distinct MT arrays through interactions with a minimal number of MT modulations. To identify such a putative central mechanism, we focused on common characteristics of neuronal MT arrays. First, developing neurites, axons, and dendrites all rely on MTs to grow¹²⁻¹⁴. Second, these neuronal compartments depend on stable MTs. In axons, the amount of stable MTs increases compared to developing neurites, and this increase is sufficient to induce axon development¹⁵. In dendrites, stable and unstable MTs are differently oriented⁹. Stable MTs are predominantly plus-end-in oriented thereby enabling cargo-sorting by directing axon-specific cargo out of dendrites⁹. Third, we and others recently found that, in developing neurites, unexpectedly, the entire MT array moves retrogradely towards the soma^{16,17}. This MT retrograde flow (MT-RF) slows down in the axon and

later also in dendrites^{16,17}. Could these similarities indicate a common mechanism driving neuronal polarity?

By building a biophysical model integrating experimental data across developmental stages, we identified slowdown of MT-RF as the unifying mechanism for the development and maintenance of axons, and dendrites. MT-RF slowdown, together with only three modulations of the MT array – stability and two types of nucleation – explains the formation of the stable MT distribution in axons and dendrites, which gives rise to polarized neuronal morphology.

Results:

Stable and unstable MTs have different distributions in developing neurites

MTs across the length of axons and dendrites have several functions. For example, distal MTs at the axon tip drive axon growth^{12,15,18–20}, while the distribution of MTs across the dendrite directs transport¹⁰. To investigate the regulation of the MT array during development, we characterized the distribution of stable and unstable MTs in developing neurites after one day in culture. We first assessed the fraction of stable MT mass by measuring the decay of MT mass after photoconversion in the proximal neurite, when MTs moved retrogradely to the cell body, referred to as MT-RF^{16,17} (Fig. 1a-c). The decay curve of photoconverted MT mass was fitted well by a dual-exponential function but not a mono-exponential function (Fig. 1d). This indicated the presence of two populations of MTs with distinct decay constants. We observed a fast-decaying component that corresponds to unstable MTs and a slow-decaying

component that corresponds to stable MTs (Fig. 1b,d). In 84% of neurites, a part of MT mass decayed slowly (stable MTs). Overall, $40 \pm 4\%$ of MT mass decayed slowly with the median half-life of 9.1 min while $60 \pm 4\%$ of MT mass decayed fast with a median half-life of 1.0 min (unstable MTs; mean $\pm$ SEM; Fig. 1e-f).

To measure the distribution of these stable and unstable MTs in neurons, we stained primary hippocampal neurons for acetylated and detyrosinated tubulin as markers for long-lived and thus stable MTs and tyrosinated tubulin as a marker for short-lived and thus unstable MTs^{15,21–23} (Fig. 1g-k; Extended Data Fig. 1a-j). Tyrosinated MTs decreased towards the neurite tip (Extended Data Fig. 1b, e-g). Interestingly, despite both being established markers for stable MTs in neurons^{11,15,24,25}, the distributions of acetylated and detyrosinated MTs differed. This difference may occur because detyrosination is slower than acetylation²⁶, raising the possibility that they may mark a different subset of MTs. Acetylated MTs decreased towards the neurite tip while detyrosinated MTs remained relatively constant in the first half of the neurite and then decreased (Extended Data Fig. 1b, e-j).

To understand which marker represents stable MTs in neurons, we analyzed the ratio of stable to unstable MTs across the neurite separately for detyrosinated and acetylated tubulin as markers for stable MTs. For acetylated tubulin as a marker for stable MTs, the corresponding marker for unstable MTs would be non-acetylated tubulin. However, since it is not possible to stain for non-acetylated tubulin, we calculated this distribution based on the other tubulin modifications. To this end, we assumed different fractions of MTs that are both acetylated and detyrosinated or acetylated and tyrosinated (Methods section “Calculating non-acetylated tubulin distribution”, Fig. 1l). Our analysis and calculations showed that the ratio of stable to unstable MTs decreased for acetylated tubulin as a marker for stable MTs towards the

neurite tip, while it increased for detyrosinated tubulin as a marker for stable MTs towards the neurite tip (Extended Data Fig. 1c).

To test which of the two ratios recapitulated the MT distributions, we measured the fraction of stable MT mass from the decay of MT mass after photoconversion in the neurite close to and far from the soma, at one third and two thirds of neurite length towards the neurite tip, respectively (Fig. 1m). The fraction of stable MT mass decreased from $34 \pm 5\%$ close to the soma to $23 \pm 5\%$ far from the soma (Fig. 1n-p), showing that the amount of stable MTs decreased towards the neurite tip relative to unstable MTs. This suggests that acetylated tubulin recapitulates stable MTs in developing neurites better than detyrosinated tubulin (Fig. 1j).

We further validated our results by directly comparing the photoconversion results to measured MT distributions by calculating the normalized change of the stable to unstable MT ratio from close to the soma to far from the soma (Fig. 1q, Extended Data Fig. 1d). This normalized change was similar to the value for acetylated tubulin but very different from detyrosinated tubulin as markers for stable MTs (-0.35 for photoconversion results, -0.25 for acetylated tubulin, 0.09 for detyrosinated tubulin) (Fig. 1q, Extended Data Fig. 1d). Therefore, we used non-acetylated and acetylated tubulin as markers for unstable and stable MTs, respectively, from here onwards (Fig. 1k,l). Taken together, the amount of unstable MTs remains relatively constant in the proximal half of the neurite and decreases distally, while the amount of stable MTs decreases along the whole neurite (Fig 1k,l). These data were the basis for uncovering how MT dynamic properties affect MT distributions in developing neurites.

Developing a biophysical model of MT stabilization in neurites

We next investigated the role of MT dynamic properties in regulating MT distributions in developing neurites from a theoretical perspective. In mitotic cells, MT arrays during cell division are well defined through biophysical models that integrate several mechanisms such as basic MT dynamics known from *in vitro* data and modulation by motor proteins^{27–33}. We hypothesized that a data-driven biophysical model will help to understand the development and maintenance of neuronal MT arrays. We therefore developed a one-dimensional model of unstable and stable MTs that undergo MT-RF (Fig. 2a-b; Methods section “Description of the core model”).

Previous models of neuronal MTs lacked MT-RF nor included the difference between stable and unstable MTs^{34–36}. To include stable and unstable MTs, our model characterizes these MTs with a long and short half-life, respectively, and describes the transitions between these states (Fig. 2b). To account for the local nucleation differences in each neuronal compartment^{37,38}, we investigated local MT distributions by incorporating three known nucleation mechanisms in neurons. New MTs can be nucleated by somatic nucleation from MT organizing centers^{37,39,40}, uniformly in the cytosol of the neurite^{41,42} or from the lattice of pre-existing MTs by MT branching^{37,42} (Fig. 2a); MT branching nucleation is limited by a global resource (Methods section “Description of the core model”). Importantly, MT-RF is modeled through all MTs moving retrogradely towards the soma at the same speed, consistent with our previous finding that the entire MT array moves uniformly in a retrograde fashion¹⁶ (Fig. 2a). Additionally, the model incorporates well known MT dynamics, including growth of unstable MTs from the tip of stable MTs and catastrophe^{43,44}. The model contains 17 parameters, of which 7 parameters were measured here, calculated, or taken from literature (Table 1; Extended Data Fig. 2a-g). The remaining

10 parameters were estimated from the comparison to 6 experimental data points and an additional 3 experimental distributions (Table 1; Methods section “Fitting parameter values”). To obtain model MT distributions, we performed Monte Carlo simulations and numerically integrated the corresponding mean-field model (Extended Data Fig. 3a-c; Methods). These MT distributions provide the basis to investigate how known MT dynamics and MT-RF interact to influence stable and unstable MTs.

MT-RF slowdown increases stable and distal MTs depending on the stable MT half-life

Since MT-RF slows down during neuronal development in the axon and later in dendrites^{16,17}, we first explored the distributions of unstable and stable MTs with fast and slow MT-RF. The number of unstable MTs remains constant close to the soma (proximal) and then decreases towards the end (distal) for both the fast MT-RF of 0.5 $\mu\text{m}/\text{min}$ measured in developing neurites¹⁶ and without MT-RF (MT-RF speed set to 0 $\mu\text{m}/\text{min}$) (Fig. 2c). In contrast, the number of stable MTs decreases across the entire region with fast MT-RF of 0.5 $\mu\text{m}/\text{min}$ (Fig. 2c). Only without MT-RF, the number of stable MTs remains constant proximally and decreases distally, similar to unstable MTs (Fig. 2c). This suggests that MT-RF slowdown increases stable MTs more strongly than unstable MTs.

Since stable MTs are key for axon development¹⁵, we next asked why MT-RF slowdown is increasing stable MTs more strongly than unstable MTs. The key difference between stable and unstable MTs is that the reaction rate which removes stable MTs – the stable MT loss rate - is lower compared to the reaction rate at which unstable MTs are removed – the catastrophe rate (Fig. 2b). Thus, we hypothesized

that increasing the stable MT loss rate results in a smaller increase of stable MTs upon MT-RF slowdown. Indeed, for a higher stable MT loss rate, MT-RF slowdown from 0.5 $\mu\text{m}/\text{min}$ to 0 $\mu\text{m}/\text{min}$ barely increases the average number of stable MTs in the entire region (global stable MTs, Fig. 2d,f). Conversely, for a lower stable MT loss rate, this MT-RF slowdown massively increases global stable MTs (Fig. 2e,f). Global unstable MTs are not changed by MT-RF for all stable MT loss rates (Fig. 2d-f). This shows that MT-RF slowdown increases stable MTs more than unstable MTs due to the lower stable MT loss rate. In other words, MT-RF moves longer lived MTs (which have a lower stable MT loss rate (Extended Data Fig. 4a,b)) further towards the soma during their longer lifetime, reducing them more. Consequently, MT-RF slowdown leads to a stronger increase for longer lived MTs.

For axon growth, in addition to stable MTs, MTs need to reach the distal tip^{12,15,19,20}. We therefore investigated the distal MT contribution in relation to MT-RF and stable MT-RF loss rates. Interestingly, for all stable MT loss rates, MT-RF slowdown from 0.5 $\mu\text{m}/\text{min}$ to 0 $\mu\text{m}/\text{min}$ increases stable and unstable MTs distally more than proximally (Fig. 2c-e, Extended Data Fig. 4c). The corresponding increase of the average number of stable and unstable MTs in the distal region (distal MTs) by MT-RF slowdown is largest for a low stable MT loss rate (Fig. 2g), leading to a high overall increase in distal MTs (Fig. 2g, "Stable + unstable"). Thus, MT-RF slowdown not only increases stable MTs but also overall distal MTs; this increase becomes stronger for lower stable MT loss rates.

MT dynamic properties explain experimental unstable and stable MT distributions

To test the predicted increase of stable and distal MTs by MT-RF slowdown, we first explored whether the interplay of modelled MT dynamics can explain experimental dynamics and distributions of stable and unstable MTs in neurons. Indeed, the model reproduces the experimental mass decay curve by adjusting only the catastrophe rate for unstable MTs and the loss rate of stable MTs while keeping the other parameters constant (Fig. 3a-d; Table 1; see Methods section “Statistics”). Using the same set of parameters (Table 1), the model reproduces the fraction of stable MT mass close to and far from the soma (Fig. 3e,f) and the distribution of stable and unstable MTs, except very close to the soma (Fig. 3g).

For additional experimental data to compare the model to, we obtained the experimental distribution of growing MT tips from live cell imaging data of fluorescently labeled end-binding protein 3 (EB3)¹⁶. Indeed, the experimental density of growing MT tips close to the soma matched the density of growing tips in the model (Fig. 3h). Further, the experimental distribution of growing MT tips remained relatively constant in the first 40% of the neurite and then decreased towards the neurite tip (Fig. 3i), which is fitted well by the model (Fig. 3i). In summary, our comparative analyses of experimental data and model predictions suggest that the modelled MT dynamics explain distributions of unstable and stable MTs as well as growing MT tips in developing neurites. This indicates that the model could be useful for making predictions about how MT-RF affects stable and unstable MTs.

MT-RF slowdown increases stable MTs distally in developing neurites

Since MT-RF slows down early in axon development¹⁶, we investigated how slow MT-RF in young axons affects stable and unstable MTs. Keeping the same set of parameters (Table 1), the model predicts that changing the MT-RF speed from 0.5 $\mu\text{m}/\text{min}$ to 0.2 $\mu\text{m}/\text{min}$, the speed in young axons after one day in culture¹⁶, increases stable MTs by 21.8% globally but not unstable MTs (Fig. 4a,b). Interestingly, in the distal region, the model predicts that both stable and unstable MTs increase noticeably with slow MT-RF, by 148.7% and 35.0%, respectively (Fig. 4b).

To validate the predicted stronger effect of MT-RF on distal MTs in developing neurites, we compared model predictions with our published experimental data for the speedup of MT-RF in developing neurites using membrane-recruited dynein¹⁶ (Fig. 4c). Using this experimental data for MT-RF speedup to $2.41 \mu\text{m}/\text{min} \pm 0.15 \mu\text{m}/\text{min}$ ¹⁶ we measured the corresponding loss of MTs. For this MT-RF speedup and using the stable MT loss rate matching the experimental stable MT half-life of approx. 10 min (Fig. 1e), the model predicts an MT loss of $44.7\% \pm 0.4\%$ globally and a higher MT loss of $88.0\% \pm 0.4\%$ in the distal region (Fig. 4d). Thus, our theoretical prediction can provide an explanation for the experimental data, where the MT loss was $52.5\% \pm 3.8\%$ in the entire neurite and also higher in the distal neurite with $74.4\% \pm 4.0\%$ (Fig. 4d). This shows that the model can account for the experimentally measured MT loss by only changing MT-RF to experimentally measured values while keeping other parameters unchanged. It suggests that the model recapitulates the experimental effect of MT-RF on MTs in developing neurites. In summary, MT-RF slowdown in developing neurites mainly increases stable MTs, particularly in the distal neurite.

MT-RF slowdown in dendrites increases stable MTs globally and distally

In dendrites, stable MTs are crucial for cargo sorting⁹. Yet, it is unclear whether stable MTs increase in dendrites during development. Additionally, MT-RF slows down in dendrites to 0.1 $\mu\text{m}/\text{min}$, albeit later than in axons¹⁶. Since we found that MT-RF slowdown increases stable MTs in developing neurites (Fig. 4), we tested whether stable MTs also increase in dendrites, and whether this is fueled by MT-RF slowdown. Interestingly, in addition to slow MT-RF, we found experimentally that the half-life of stable MTs is longer in dendrites than in developing neurites (Fig. 5a,b). We lower the stable MT loss rate in the model to match this higher experimental stable MT half-life (Fig. 5c). To model MT dynamics in the dendrite, we use this lower stable MT loss rate and slower MT-RF speed but otherwise keep the same set of parameters as for developing neurites (Table 1). The model predicts that these two changes together allow dendrites to increase the amount of stable MTs globally by 203.6% and distally by 408.8%, compared to the scenario with higher stable MT loss rate and faster MT-RF, as in neurites (Fig. 5d,e). This stable MT increase results in a stable MT mass fraction of $59.3\% \pm 0.7\%$ in the middle of the dendrite, which is similar to the experimentally measured value of $57.8\% \pm 4.8\%$ (Fig. 5f). This shows that dendrites massively increase stable MTs, transport roads for dendritic cargo⁹, by only slowing down MT-RF and lowering the stable MT loss rate.

To dissect the mechanisms underlying the increase in stable MTs, we tested the influence of MT-RF slowdown and low stable MT loss rate separately. The model predicts that with slow MT-RF of 0.1 $\mu\text{m}/\text{min}$, lowering the stable MT loss rate increases the amount of stable MTs globally and distally, by 158.0% and 63.5%, respectively (Fig 5g,h). In contrast, with fast MT-RF of 0.5 $\mu\text{m}/\text{min}$, lowering the stable MT loss rate increases the amount of stable MTs only by 69.0% globally and reduces

distal stable MTs by 9.5% (Fig. 5g,h). Thus, these results suggest that dendrites can maintain a high amount of stable MTs globally and distally by synergistically reducing the stable MT loss rate and slowing down MT-RF.

We validated the predicted effect of MT-RF on MTs in dendrites by comparing model predictions with our published experimental data for MT-RF speedup in dendrites using membrane-recruited dynein¹⁶ (Fig. 5i). Using these experimental data with MT-RF speedup to $1.39 \mu\text{m}/\text{min} \pm 0.3 \mu\text{m}/\text{min}$ ¹⁶ we measured the corresponding MT loss in dendrites. For this MT-RF speedup and using the same parameter values as in dendrites (Table 1), the model predicts a global MT loss after MT-RF speedup of $51.2 \% \pm 0.2\%$ and a higher distal MT loss of $91.5\% \pm 0.2\%$ (Fig. 5j). This matches the experimental global MT loss of $55.7\% \pm 7.0\%$ and the higher experimental distal MT loss of $84.6\% \pm 4.3\%$ (Fig. 5j). Hence, it demonstrates that changing only MT-RF speed to experimentally measured values can explain the experimentally measured MT loss. Further, it shows that the model makes validated predictions about dendrites by only changing the two parameters stable MT loss rate and MT-RF speed while keeping the other parameters used to model developing neurites unchanged (Table 1). In summary, with slow MT-RF, increasing stable MT half-life leads to more global and distal stable MTs. In contrast, with faster MT-RF this increase of global stable MTs is reduced and the increase of distal stable MTs reverses to a decrease. Thus, dendrites need to slow down MT-RF in addition to increasing stable MT half-life to maintain a high amount of stable MTs.

MT-RF slowdown in axons allows MTs to increase towards the axon tip

Slowdown of MT-RF is a key step for axon development^{16,17}. Additionally, stable MTs are known to be crucial for axons, with MT stabilization being sufficient for axon development¹⁵. In particular, distal MTs are crucial for fast axonal growth^{12,15,19,20}. The consistent increase of stable and distal MTs by MT-RF slowdown in developing neurites and dendrites prompted us to investigate whether MT-RF slowdown also increases stable and distal MTs in axons. We first tested whether the model can help to understand the formation of the MT array in axons. Axons differ from developing neurites and dendrites^{3,20,45}. We focus on the following two key differences (Fig. 6a): longer shaft length of 160 μm , compared to 30-40 μm used for developing neurites and dendrites, and lower cytosolic nucleation rate⁴². We also measured three additional differences experimentally: 0.1 $\mu\text{m}/\text{min}$ slow MT-RF (Extended Data Fig. 5a-c), longer stable MT half-life (Fig. 6b-d) and a higher fraction of stable MT mass (Fig. 6e). These five differences are implemented in the model by changing five parameters, while keeping the other parameters as in developing neurites and without adding additional mechanisms to the model (Table 1). To fit the model to the longer stable MT half-life and higher fraction of stable MT mass, we lower the stable MT loss rate and increase the stabilization rate (Fig. 6f,g). The lower cytosolic nucleation rate is key to obtain a model that is in line with the uniform plus-end-out MT orientation in axons⁵ (Extended Data Fig. 6a-c). Specifically, MT branching allows newly nucleated MTs to have the same orientation as the MT from which they were nucleated^{46,47} (Extended Data Fig. 6a) and thereby does not directly determine MT orientation. Conversely, nucleation from the soma leads to plus-end-out MTs (Extended Data Fig. 6a). Lastly, MTs nucleated in the cytosol of the axon have an equal chance to be plus-end-out or plus-end-in (Extended Data Fig. 6a),

which reduces the percentage of plus-end-out MTs in axons^{42,46}. This suggests that cytosolic MT nucleation is kept low in the axon to allow uniform plus-end-out MT orientation⁴². Without a reduction of the cytosolic nucleation rate, the percentage of plus-end-in MTs in the model is $43.3 \pm 0.1\%$ and thus much higher than the experimentally measured value of 2.5% (Extended Data Fig. 6b). To obtain a realistic level of cytosolic nucleation rate for the axon, we reduce this nucleation rate to achieve a percentage of plus-end-in MTs of 2.8%, close to experimentally measured value of 2.5%⁵ (Extended Data Fig 6c).

Since MT-RF slows down in axons compared to developing neurites, we first tested the effect of slow MT-RF compared to fast MT-RF on stable and unstable MT distributions. With the parameter changes noted above and slow MT-RF of 0.1 $\mu\text{m}/\text{min}$, the model predicts that the number of stable and unstable MTs decreases towards the distal region (Fig. 6i; low resources for MT branching). Since MT branching is more prevalent in axons than in developing neurites^{42,46} and since a reduction of MT branching impairs axon development⁴², we tested the effect of increased MT branching. In our model, MT branching is mainly limited by the amount of available resources. With higher amounts of resources for MT branching, but keeping other parameters unchanged, the number of stable and unstable MTs increases towards the axon tip for slow MT-RF of 0.1 $\mu\text{m}/\text{min}$ (Fig. 6h,i; high resources for MT branching). In contrast, with fast MT-RF of 0.5 $\mu\text{m}/\text{min}$, the number of stable and unstable MTs decreases towards the axon tip even with higher amounts of resources for MT branching (Fig. 6h,j). This suggests that MT-RF slowdown allows the higher MT branching in axons^{42,46} to increase distal MTs (Fig. 6h). Thus, MT-RF slowdown in axons not only directly increases distal MTs but also enables the increase of distal MTs by increased MT branching.

We next wanted to test our prediction that stable MTs in axons increase towards the axon tip. To this end, we stained neurons with an older axon after three days in culture for tyrosinated tubulin as a marker for younger MTs as well as acetylated and detyrosinated tubulin as markers for older MTs (Extended Data Fig. 7a-e). As for developing neurites (Fig. 1g-l, Extended Data Fig. 1a-j), we used the distribution of acetylated tubulin as a marker for stable MTs and calculated the distribution of non-acetylated tubulin to obtain the distribution of unstable MTs (Extended Data Fig. 7a-e). The resulting distributions for stable and unstable MTs matched the change of the ratio of stable to unstable MTs from close to the soma to far from the soma, measured by live cell imaging of MT decay (Extended Data Fig. 7f-i). The amount of unstable MTs decreases in the proximal half of the axon and then increases (Fig. 6k). Importantly, the amount of stable MTs increases until close to the axon tip (Fig. 6k). The unstable MT distribution cannot be fit well by the model (Fig. 6k), particularly close to the soma, arguing for additional molecular mechanisms that play a role in this region, such as the emerging axonal initial segment (AIS)^{45,48}. Notwithstanding, the distribution of stable MTs is fit well by the model throughout the axon (Fig. 6k). This supports the prediction that distal stable MTs are increased in axons through a combination of MT-RF slowdown and increased MT branching (Fig. 6h-j).

MT-RF slowdown maintains distal MTs in axons while allowing high stable MT half-life and uniform plus-end-out MTs

Since distal MTs are required for fast axonal growth^{12,15,19,20}, we investigated the mechanisms regulating distal MTs in axons. First, we characterized the role of axonal MT-RF slowdown for distal MTs by performing simulations with different MT-RF speeds (Fig. 7a). The model predicts that without MT-RF slowdown in the axon,

using the MT-RF speed of 0.5 $\mu\text{m}/\text{min}$ in developing neurites, barely any distal unstable or stable MTs are left (Fig. 7b-d), leaving the distal axon without MT: the MT array is only 0.7 ± 0.0 MTs thick (Fig. 7d). Only with MT-RF speeds below 0.3 $\mu\text{m}/\text{min}$, some distal MTs remain (Fig. 7d). Further slowdown from the speed in young axons of 0.2 $\mu\text{m}/\text{min}$ to the speed in old axons of 0.1 $\mu\text{m}/\text{min}$ is also relevant since it increases distal MTs two-fold (Fig. 7d). This suggests that MT-RF slowdown is needed to maintain distal MTs in axons. While the increase of distal MTs by MT-RF slowdown in axons (Fig. 7b,c) is consistent with our results in developing neurites (Fig. 4) and dendrites (Fig. 5), it is much stronger in axons. Therefore, axons are particularly dependent on MT-RF slowdown to maintain distal MTs. This raises the question of which axon-specific mechanisms lead to the low number of distal MTs without MT-RF slowdown.

Since stable MTs are key for axonal development¹⁵, reducing the stable MT loss rate to increase stable MTs is an important mechanism for axons. Therefore, we second tested the role of the lower stable MT loss rate, and thus the longer stable MT half-life (Fig. 6f) in axons (Fig. 7e), compared to the higher stable MT loss rate in developing neurites (Figs. 3-4). The model predicts that without MT-RF slowdown, lowering the stable MT loss rate to the axonal value massively reduces both distal unstable and distal stable MTs, so that barely any distal MTs are left (Fig. 7f-h). In contrast, with the slow MT-RF speed of 0.1 $\mu\text{m}/\text{min}$ in old axons, lowering the stable MT loss rate hardly reduces distal unstable MTs and even increases distal stable MTs (Fig. 7g,h). Thus, lowering the stable MT loss rate in axons requires MT-RF slowdown to prevent loss of distal MTs. This also shows that a lower stable MT loss rate is not only a key mechanism to increase stable MTs but also allows MT-RF to tightly regulate distal axonal MTs.

Third, we tested the role of uniform plus-end-out MT orientation (Fig. 7i), which is a key property of axons to enable axonal transport and cargo sorting⁸. To investigate the role of uniform plus-end-out MT orientation, we tested the effect of lowering the cytosolic nucleation rate from the value used in developing neurites and dendrites (Figs. 3-5) to the much lower value fitted to the high plus-end-out MT fraction in axons (Fig. 6). Lowering the cytosolic nucleation rate without MT-RF slowdown massively reduces distal MTs, from 16.2 ± 0.2 MTs to 0.8 ± 0.1 MTs (Fig. 7j-l). For slow MT-RF, lowering the cytosolic nucleation rate does not change distal MTs noticeably (Fig. 7j-l). This suggests that uniform plus-end-out MT orientation makes MT-RF slowdown necessary to retain distal MTs and thereby maintain axonal length. In conclusion, the model suggests that slowdown of MT-RF together with its interplay with two axon-specific changes is important for maintaining long axons and fast axon growth through retaining distal MTs. In other words, MT-RF needs to slow down in axons to maintain distal MTs, while also keeping a high fraction of stable MTs and uniform plus-end-out MTs.

In summary, by integrating experimental data on MT dynamics and MT distributions in a biophysical model we uncover the central role of MT-RF slowdown for axons and dendrites (Fig. 7m). In dendrites, MT-RF slowdown enables a large increase in stable MTs. In axons, MT-RF slowdown is necessary for MTs to reach the axon tip. The strong effects of MT-RF slowdown in dendrites and axons are caused by increased stable MT half-life; in the axon, this effect is even further augmented due to the uniform MT orientation. The interaction of MT-RF slowdown with these two MT properties and MT branching is sufficient to explain the changes in the stable MT distribution for axon and dendrite development.

Discussion

So far, axon and dendrite development have been thought to require different mechanisms in axons and dendrites to trigger morphological and molecular polarization². Our data overturn this view by showing that the same mechanism, slowdown of MT-RF, enables development and maintenance of the axon and dendrites. By investigating the distribution of stable and unstable MTs we reveal how distinct MT dynamics in axons and dendrites drive changes in stable MTs, required for polarized transport, and distal MTs that promote axon growth. We find that the interaction of MT-RF with only three MT dynamics - MT stability, cytosolic MT nucleation and MT branching - is sufficient to explain the change of the stable MT distribution for both axon and dendrite development. Our unifying perspective leads to four unexpected insights.

First, neurite growth is thought to increase through the regulation of distal MTs by the growth cone^{49,50}. Here, we find that in addition distal MTs are strongly regulated by MT-RF acting on the entire MT array to redistribute MTs away from the neurite tip. This redistribution reduces neurite growth, which is supported by MT-RF speedup inducing neurite retraction¹⁶. After MT-RF slowdown, increased MT branching in axons⁴² allows axons to increase their distal MTs. Interestingly, reduced MT branching through HAUS7 knockout reduces axon regeneration⁵¹. This emphasizes a new avenue of looking beyond growth cone-mediated mechanisms towards axon shaft-mediated mechanisms that can redistribute MTs towards the growth cone to increase axon growth.

Second, while axons are known to require an increase in stable MTs¹⁵, dendrites are mainly characterized as having fewer stable MTs than axons^{52–54}. While the fraction of stable MTs increases among minus-end-out oriented MTs during dendrite development^{9,55}, it has remained unexplored whether overall stable MTs increase or whether stable MTs only change their orientation⁵⁵. We now find that dendrites massively increase stable MTs. Previously, knockout of the MT stabilizing protein MAP2 in Purkinje cells led to a 23% loss of MT density in dendrites¹⁴. While this was discounted as being a minor decrease⁵⁶, it matches our predicted MT loss of 28% for decreasing the dendritic stable MT half-life to the level of developing neurites and in fact corresponds to a large predicted stable MT loss of 60%. This supports the role of increased stable MTs in dendrites and highlights the insights gained when comparing experimental results with model predictions.

Third, it has remained unclear which cell-inherent mechanism allows dendrites to switch from the non-growing state of developing neurites to a growing state. Interestingly, KO of the MT stabilizing protein MAP2 reduces dendritic length¹⁴, suggesting that stable MTs are needed for dendrite growth, as known in axons. Nonetheless, it was thought that a potential trigger for dendritic growth would be based on different mechanisms than axonal growth⁵⁷. Instead, we found that the same mechanism that triggers stable axon growth could also allow dendrites to grow through increasing distal MTs and stable MTs: slowdown of MT-RF. This further highlights the unifying features of our model centering around the MT-RF.

Lastly, intuitively one would assume MT stabilization should be sufficient to increase stable MTs for axon and dendrite development. However, we find that MT-RF suppresses the stabilization-mediated increase in stable MTs by reducing stable MTs with a longer half-life even more. Thus, MT-RF slowdown is needed in addition to MT stabilization for developmentally relevant increases in stable MTs. This can also explain how MT-RF slowdown in the whole neuron directs Kinesin-1 to all neurites and subsequently leads to the development of multiple axons¹⁷. Before axon development, stable MTs stochastically increase in single neurites, probably mediated by increasing stable MT half-life, which recruits the MT motor protein Kinesin-1 into the neurite through more detyrosinated MTs^{24,58,59}. However, with fast MT-RF, the increase in stable MTs - driven by their extended half-life - is attenuated, which could explain its transient nature in developing neurites⁵⁸. In contrast, MT-RF slowdown allows a steady increase in stable MTs, stabilizing Kinesin-1 recruitment and axon identity.

Why does early MT-RF slowdown lead to axon development, whereas later it leads to dendrite development? The key difference is the orientation of MTs that are stabilized. Axonal cargo is steered into axons through detyrosinated, stable MTs which are plus-end-out oriented⁵⁹. Thus, if plus-end-out MTs are stabilized, then an axon forms. In dendrites on the other hand stable MTs keep axonal cargo out, through changing their orientation to plus-end-in^{9,55}. Therefore, if stabilized MTs change their orientation to plus-end-in then a dendrite forms.

Intriguingly, tuning of MT flow could be a conserved mechanism to regulate length depending on MT half-life across different biological contexts. During cell division, MTs move towards the poles of the mitotic spindle at around $0.6 \mu\text{m}/\text{min}$ ⁶⁰, which slows down in anaphase^{61,62} to enable spindle elongation⁶³. This is remarkably similar to MT-RF in neurons, which moves MTs retrogradely at $0.5 \mu\text{m}/\text{min}$ and slows down in axons and dendrites^{16,17}, which we found in this study to enable axon and dendrite elongation. As in neuronal development, also during cell division, the half-life of MTs changes in different phases, which regulates chromosomal instability in cancer cells⁶⁴. MT half-life in anaphase can even become as high as in dendrites⁶². Thus, the half-life dependent effect of MT-RF we found in neurons could also be relevant for MT flux during cell division. Thus, our model with MT stabilization could be employed to investigate the effect of MT stability during cell division.

Importantly, the MT cytoskeleton is perturbed in several neurodevelopmental diseases^{65–67}, neurodegenerative diseases^{68–70} and spinal cord injury^{71,72}, often showing MT loss, changes in MT stability or changes in tubulin acetylation. However, which changes in MT dynamics are sufficient to cause these pathological phenotypes has remained unclear. Interestingly, for several neurodevelopmental⁶⁶ and neurodegenerative diseases⁷³, as well as spinal cord injury^{18,74}, mild MT stabilization improves the condition. While the similarities we uncovered between axons and dendrites suggest that MTs could be pathologically changed in both axons and dendrites, our data also highlight that the same treatment may lead to different outcomes in axons and dendrites. Our data-driven biophysical model would allow to systematically characterize which experimentally measured changes in MT dynamics are sufficient to explain pathological changes in MT distributions in axons and

514 dendrites. This could provide mechanistic avenues towards finding combinations of
515 MT dynamics that can be changed to combat disease.

Online Methods

Description of the core model

We developed a one-dimensional model of MTs in the neurite, where the origin of the coordinate system is located at the soma and the neurite tip is at $x = L$. Each MT consists of a stable and unstable part of lengths l_s and l_u , respectively. The position of the MT, x , represents the position of the minus-end of the MT, while the sum of the position x , the stable length l_s , and the unstable length l_u represents the position of the plus-tip of the MT. Based on the dynamic properties of MTs, the model describes three key populations of MTs: those that are (i) unstable and growing, (ii) unstable and pausing, and (iii) stable (Fig. 2a-b). In the model, all MTs have their plus-end oriented towards the neurite tip (plus-end-out), while in neurons only 80% of MTs are oriented in this way⁵. MT stabilization is modeled as a stabilization of the MT tip and thus is a transition from an unstable MT to a stable MT. To describe how the density of unstable growing MTs plus ends changes in time, t , we describe their dynamics by a transport equation:

$$\frac{\partial \rho_g(x, l_s, l_u)}{\partial t} = \delta(l_u)k_{\text{nuc}} + \delta(l_u)k_{\text{growth}}\rho_s + \frac{\partial v_f \rho_g}{\partial x} - \frac{\partial v_g \rho_g}{\partial l} + k_{\text{res}}\rho_p - k_{\text{pause}}\rho_g \quad A1$$

Here, flow velocity, growth velocity, pausing rate (transition from the growing to the pausing state) and rescue rate (transition from the pausing to the growing state) are denoted v_f , v_g , k_{pause} and k_{res} , respectively. The growth rate is the transition from the stable state to the growing state and thus represents the growth of an unstable MT from the tip of a stable MT and is denoted k_{growth} . This leads to the formation of well-

known MTs that are stable towards the minus-end and unstable towards the plus-end^{43,44} (Fig. 2b). The nucleation rate, $k_{\text{nuc}} = k_0\delta(x) + k_{\text{cyto}} + k_{\text{branch}} \cdot n \cdot \left(1 - \frac{N}{N_{\text{max}}}\right)$, includes nucleation from the soma with rate k_0 , from the cytosol with rate k_{cyto} , and along pre-existing MTs with rate k_{branch} . The number of microtubules along the neurite is given as $n = \int_0^x \int_{x-\tilde{x}}^{L-\tilde{x}} (\rho_s + \int_0^{L-x-l_s} (\rho_g(x, l_s - l_u, l_u) + \rho_p(x, l_s - l_u, l_u)) dl_u) dl_s d\tilde{x}$, and the total number of microtubules in the neurite is $N = \int_0^L \int_0^{L-x} (\rho_s + \int_0^{L-x-l_s} (\rho_g + \rho_p) dl_u) dl_s dx$. The number of available resources for nucleation along pre-existing MTs is denoted N_{max} . δ is the dirac delta function.

To describe how the density of unstable pausing MT plus ends changes in time t , we describe their density by another transport equation:

$$\frac{\partial \rho_p(x, l_s, l_u)}{\partial t} = \frac{\partial v_f \rho_p}{\partial x} + k_{\text{pause}} \rho_g - (k_{\text{res}} + k_{\text{cat}} + k_{\text{stable}}) \rho_p. \quad A2$$

The catastrophe rate (loss of the unstable length of the MT l_u) and stabilization rate (the transition from the pausing unstable state to the stable state) are denoted k_{cat} and k_{stable} , respectively. The other parameters are described above.

Lastly, to describe how the density of stable MT plus ends changes in time t , we describe their density by the following equation:

$$\begin{aligned} \frac{\partial \rho_s(x, l_s)}{\partial t} = & \frac{\partial v_f \rho_s}{\partial x} + k_{\text{stable}} \rho_p|_{l_u=l_s} - (k_{\text{loss}} + k_{\text{growth}}) \rho_s + k_{\text{cat}} \int_0^{L-x-l_s} \rho_p dl_u \\ & + k_{\text{stable}} \int_0^{l_s} \rho_p(x, l_s - l_u, l_u) dl_u. \end{aligned} \quad A3$$

The loss rate (complete loss of the stable MT) is denoted as k_{loss} . The other parameters are as described above. The integral for the catastrophe of pausing unstable MTs $k_{\text{cat}} \int_0^{L-x-l_s} \rho_p dl_u$ represents the loss of the unstable length l_u of the

pausing unstable MT ρ_p , after which only the stable length l_s remains. Thus, the density ρ_p is integrated across all MTs with the same stable length l_s at position x . The integral for the stabilization of pausing unstable MTs $k_{\text{stable}} \int_0^{l_s} \rho_p(x, l_u, l_s - l_u) dl_u$ represents the stabilization of the unstable length l_u of the pausing unstable MT ρ_p to stable length l_s . Thus, the density ρ_p is integrated along the line of MTs with the same total length $l_s + l_u$ with their minus end at the same position x .

This set of equations represent the core of the model. Additionally, changes at the boundary of the neurite tip have to be taken into account.

Analytical mean-field solutions

We first solve a simplified system of partial differential equations (Extended Data Fig. 3b), with flow velocity v_f , nucleation from the soma k_0 and nucleation from preexisting MTs k_{branch} set to zero. We also set the growth rate k_{growth} to zero. As a consequence, there is no growth of unstable MTs from the tip of stable MTs and unstable MTs do not have a stable length.

The change in the density of growing unstable MT plus-ends over time is described by the following transport equation:

$$\frac{\partial \rho_G(x, l)}{\partial t} = \delta(l) k_{\text{cyto}} - \frac{\partial}{\partial l} v_g \rho_G + k_{\text{rescue}} \rho_P - k_{\text{pause}} \rho_G. \quad B1$$

The nucleation rate from the cytosol, MT growth velocity, the rescue rate, and pausing rate are denoted as v_g , k_{rescue} , k_{pause} , and k_{cyto} , respectively. Here, δ is the dirac delta function.

The change in the density of pausing unstable MT plus-ends over time is described by the following equation:

$$581 \quad \frac{\partial \rho_P(x, l)}{\partial t} = -k_{\text{rescue}}\rho_P + k_{\text{pause}}\rho_G - k_{\text{stable}}\rho_P - k_{\text{cat}}\rho_P. \quad B2$$

582 The stabilization and catastrophe rate are denoted as k_{stable} and k_{cat} , respectively.

583 The change in the density of stable MT plus-ends over time is described by the
584 following equation:

$$585 \quad \frac{\partial \rho_S(x, l)}{\partial t} = k_{\text{stable}}\rho_P - k_{\text{loss}}\rho_S. \quad B3$$

586 The loss rate is denoted as k_{loss} .

587 To solve the steady state, all derivatives with respect to time are set to zero:

$$588 \quad 0 = -\frac{\partial}{\partial l} v_g \rho_G + k_{\text{rescue}}\rho_P - k_{\text{pause}}\rho_G, \quad B4$$

$$589 \quad 0 = -k_{\text{rescue}}\rho_P + k_{\text{pause}}\rho_G - k_{\text{stable}}\rho_P - k_{\text{cat}}\rho_P, \quad B5$$

$$590 \quad 0 = k_{\text{stable}}\rho_P - k_{\text{cat}}\rho_S. \quad B6$$

591 Solving the algebraic equation (B5) leads to the solution for ρ_P :

$$592 \quad \rho_P = \frac{k_{\text{pause}}}{k_{\text{rescue}} + k_{\text{cat}} + k_{\text{stable}}} \rho_G. \quad B7$$

593 Solving the algebraic equation (B6) leads to the solution for ρ_S :

$$594 \quad \rho_S = \frac{k_{\text{stable}}}{k_{\text{loss}}} \rho_P. \quad B8$$

595 Plugging equation (B7) in equation (B4) leads to:

$$596 \quad 0 = -\frac{\partial}{\partial l} v_g \rho_G + \frac{k_{\text{rescue}} k_{\text{pause}}}{k_{\text{rescue}} + k_{\text{cat}} + k_{\text{stable}}} \rho_G - k_{\text{pause}}\rho_G. \quad B9$$

597 The general solution of this first order ordinary differential equation is:

$$\rho_G = C * e^{-\left(\frac{k_{\text{pause}}}{v_g} \left(1 - \frac{k_{\text{rescue}}}{k_{\text{rescue}} + k_{\text{cat}} + k_{\text{stable}}}\right)\right)l}. \quad B10$$

To obtain the boundary condition at $l = 0$, the steady state ODE is integrated around zero:

$$0 = \int_{-\varepsilon}^{\varepsilon} \left(-\frac{\partial}{\partial l} v_g \rho_G + k_{\text{rescue}} \rho_P - k_{\text{pause}} \rho_G + \delta(l) k_0 \rho_G\right) dl. \quad B11$$

This shows that:

$$\rho_G(l = 0) = \frac{k_0}{v_g}. \quad B12$$

Consequently, the steady state solution for ρ_G is:

$$\rho_G = \frac{k_0}{v_g} * e^{-\left(\frac{k_{\text{pause}}}{v_g} \left(1 - \frac{k_{\text{rescue}}}{k_{\text{rescue}} + k_{\text{cat}} + k_{\text{stable}}}\right)\right)l}. \quad B13$$

Numerical mean-field solutions

To numerically solve the steady state of the two systems of partial differential equations in Extended Data Fig. 3a,c, a custom implementation of the finite difference algorithm with a forward Euler method is used. The code is implemented using the Python package numba, to just-in-time compile the code to run fast on the central processing unit (CPU).

For Extended Data Fig. 3a we set both the nucleation from the soma k_0 and nucleation from preexisting MTs k_{branch} to zero. Then we solve the steady state of the following system of partial differential equations, also accounting for differences at the boundary of the neurite tip.

The change in the density of unstable MT plus ends changes in time t is described by the following transport equation:

$$\frac{\partial \rho_{Gu}(x, l_u)}{\partial t} = \delta(l_u)k_{cyto} + \frac{\partial}{\partial x} v_f \rho_{Gu} - \frac{\partial}{\partial l} v_g \rho_{Gu} + k_{rescue} \rho_{Pu} - k_{pause} \rho_{Gu}. \quad C1$$

The nucleation rate from the cytosol, flow velocity, MT growth velocity, rescue rate and pausing rate are denoted as k_{cyto} , v_f , v_g , k_{rescue} and k_{pause} , respectively. Once again, δ represents the dirac delta function.

The change in the density of pausing unstable MT plus-ends over time t is described by the following transport equation:

$$\frac{\partial \rho_{Pu}(x, l_u)}{\partial t} = \frac{\partial}{\partial x} v_f \rho_{Pu} + k_{pause} \rho_G - (k_{rescue} + k_{cat} + k_{stable}) \rho_{Pu} + \delta(L - x - l_u) k_{pause} \rho_{Bu}. \quad C2$$

The catastrophe rate and stabilization rate and denoted as k_{cat} and k_{stable} , respectively.

At the boundary of the neurite tip, the change of the density of unstable growing MT plus-tips over time t is described by the following transport equation:

$$\frac{\partial \rho_{Bu}(x)}{\partial t} = \frac{\partial}{\partial x} v_f \rho_{Bu} - k_{pause} \rho_{Bu} + v_g \rho_{Gu}(l_u = L - x). \quad C3$$

The change in the density of plus-ends of stable MTs over time t is described by the following transport equation:

$$\begin{aligned} \frac{\partial \rho_S(x, l_s)}{\partial t} = & \frac{\partial}{\partial x} v_f \rho_S + k_{stable} \rho_{Pu}(l_u = l_s) - (k_{loss} + k_{growth}) \rho_S \\ & + k_{cat} \int_0^{L-x-l_s} \rho_{Psu} dl_u + k_{stable} \int_0^{l_s} \rho_{Pu}(x, l_s - l_u, l_u) dl_u. \end{aligned} \quad C4$$

The change in the density of unstable MT plus ends growing from stable MTs over time t is described by the following transport equation:

$$\frac{\partial \rho_{Gs}(x, l_u, l_s)}{\partial t} = \delta(l_u) k_{\text{growth}} \rho_S + \frac{\partial}{\partial x} v_f \rho_{Gs} - \frac{\partial}{\partial l_u} v_g \rho_{Gs} + k_{\text{rescue}} \rho_{\text{Psu}} - k_{\text{pause}} \rho_{Gs}. \quad C5$$

The change in the density of growing unstable MT plus ends growing from stable MTs over time t is described by the following transport equation:

$$\frac{\partial \rho_{\text{Psu}}(x, l_u, l_s)}{\partial t} = \frac{\partial}{\partial x} v_f \rho_{\text{Psu}} + k_{\text{pause}} \rho_{Gs} - (k_{\text{rescue}} + k_{\text{cu}} + k_{\text{Psu}}) \rho_{\text{Psu}} + \delta(L - x - l_s - l_u) k_{\text{pause}} \rho_{Bs}. \quad C6$$

At the boundary of the neurite tip, the change of the density of unstable growing MT plus-tips growing from stable MTs over time t is described by the following transport equation:

$$\frac{\partial \rho_{Bs}(x, l_s)}{\partial t} = \delta(l_u = L - x - l_s) v_g \rho_{Gs} + \frac{\partial}{\partial x} v_f \rho_{Bs} - k_{\text{pause}} \rho_{Bs}. \quad C7$$

We then solved the steady state of a simplified system of partial differential equations (Extended Data Fig. 3c) numerically with the nucleation rate from pre-existing MTs k_{branch} not set to zero but no resource limit for this nucleation. However, we set flow velocity v_f and nucleation from the soma k_0 to zero. We also set the growth rate k_{growth} to zero. As a consequence, there is no growth of unstable MTs from the tip of stable MTs and unstable MTs do not have a stable length.

The change in the density of growing unstable MT plus ends changes in time t , is described by the following transport equation:

$$\frac{\partial \rho_G(x, l)}{\partial t} = -\frac{\partial}{\partial l} v_g \rho_G + k_{\text{rescue}} \rho_P - k_{\text{pause}} \rho_G + \delta(l) k_{\text{branch}} n. \quad D1$$

The MT growth velocity, rescue rate, pausing rate and nucleation rate from preexisting MTs are denoted as v_g , k_{rescue} , k_{pause} , and k_{branch} . The density of pausing unstable MT plus-ends and stable MT plus-ends are denoted as ρ_P and ρ_S . δ is the dirac delta

function. The number of microtubules along the neurite is given as $n =$

$$\int_0^x \int_{x-\tilde{x}}^{L-\tilde{x}} (\rho_G(x, l_s) + \rho_P(x, l_s) + \rho_S) dl_s d\tilde{x}.$$

The change in the density of pausing unstable MT plus ends changes in time t , is described by the following equation:

$$\frac{\partial \rho_P(x, l)}{\partial t} = -k_{\text{rescue}}\rho_P + k_{\text{pause}}\rho_G - k_{\text{stable}}\rho_P - k_{\text{cat}}\rho_P. \quad D2$$

The stabilization rate and catastrophe rate are denoted as k_{stable} and k_{cat} .

The change in the density of stable MT plus ends changes in time t , is described by the following equation:

$$\frac{\partial \rho_S(x, l)}{\partial t} = k_{\text{stable}}\rho_P - k_{\text{loss}}\rho_S. \quad D3$$

The loss rate is denoted as k_{loss} .

Running stochastic simulations

MTs in neurites are simulated with a defined dimension, corresponding to the neurite length. Position $x = 0$ corresponds to the start of the neurite at the soma and $x = L$ corresponds to the neurite tip. MTs are not allowed to extend beyond the neurite tip, but the minus-end is allowed to move out of the neurite. MTs are simulated with a property for the position of the minus end, one property for its unstable and one property for its stable length. MTs are removed from the simulation through MT-RF reducing their position property, when their plus-end would be more than $d_{\text{soma}}^{\text{removal}}$ out of the neurite, which was set at 5 μm and thus a position lower than -5. This distance represents MTs being completely depolymerized when reaching the middle of the soma. Once MTs are removed from that position, if they were nucleated by MT branching, the corresponding resource is available again.

Stochastic simulations are run with a custom-implementation of the exact next reaction Stochastic Simulation Algorithm (SSA)⁷⁵. The algorithm is implemented using the Python package numba, to just-in-time compile the code to run many simulations in parallel on a graphics processing unit (GPU). A Python package with an application programming interface (API) was written to make stochastic simulations on GPUs easily useable (<https://github.com/maxschelski/cytostoch>). Importantly, due to approximate calculations of MT density for MT branching and of the position-dependent unstable MT loss rate (see below), simulations of the complete system are not exact. Parameter values are fitted by manually comparing simulation results to experimental data.

Due to the importance of MTs close to the neurite tip^{12,15,19,20} the neurite tip is explicitly modeled by increasing the unstable MT loss rate close to the neurite tip²⁴. The increased position-dependent unstable MT loss rate is calculated as follows:

$$k_{\text{cat}}(x) = k_{\text{cat}}^{\text{base}} + H(x + k_{\text{cat}}^{\text{dist}} - L) \cdot (x + k_{\text{cat}}^{\text{dist}} + L) \cdot k_{\text{cat}}^{\text{change}}. \quad E1$$

The baseline catastrophe rate, the change of the catastrophe rate per μm distance from the tip, and the maximum distance from the tip for which the catastrophe rate should be changed are denoted as $k_{\text{cu}}^{\text{base}}$, $k_{\text{cu}}^{\text{change}}$ and $k_{\text{cat}}^{\text{dist}}$. This results in an unstable MT loss rate that is constant throughout the neurite and linearly increases in the last micrometer to its maximum. The Heaviside function is denoted as H .

The total MT branching rate is calculated based on the following equation:

$$k_{\text{branch}}^{\text{total}} = \sum_{i=0}^{\frac{L}{x_{\text{step}}}-1} k_{\text{branch}} \cdot l_{\text{MT}}(i) \cdot \left(1 - \frac{N_{\text{branch}}}{N_{\text{max}}}\right). \quad E2$$

With i being the number of the step in x , and x_{step} is the step size, set as $1 \mu\text{m}$ (for $30 \mu\text{m}$ and $40 \mu\text{m}$ long neurites) up to $4 \mu\text{m}$ (for $160 \mu\text{m}$ long neurites). The rate for nucleation from pre-existing MTs is denoted as k_{branch} . The total MT length from the i step to the $i + 1$ step is denoted as $l_{\text{MT}}(i)$ and is calculated by summing the length of all MTs in this x range. The total number of MTs nucleated by branching and the maximum number of MTs nucleated by branching are denoted by N_{branch} and N_{max} , respectively.

Since MT length and MT position are changing, the reaction rate $k_{\text{branch}}^{\text{total}}$ is a function of time t and thus the rate λ is a function of time t as well:

$$\lambda(t) = k_{\text{branch}}^{\text{total}}(t) + \sum_{i=0}^I k_i. \quad E4$$

The sum of all reaction rates other than branching nucleation rate is denoted as $\sum_{i=0}^I k_i$, with I being the total number of other reaction rates.

Thus, the following integral needs to be solved to obtain Tau:

$$\lambda_{\text{total}}(\tau) = \int_0^{\tau} \lambda(t) dt. \quad E5$$

Tau is obtained as a random number, R drawn from an exponential distribution, divided by the total rate λ_{total} :

$$\tau = \frac{-\log(R)}{\lambda_{\text{total}}(\tau)}. \quad E6$$

Due to the nonlinear Heaviside step functions due to absolute limits on MT growth, this integral is solved by iteratively obtaining better guesses of Tau. The first guess of Tau is based on the current sum of all rates, ignoring the time dependence. For each

guess, the integral equation is solved, obtaining a value for the rate $\lambda_{total}(\tau)$ and thus a new value for Tau. The guess is therefore adjusted by moving it a bit closer to the new Tau. This is done for a maximum of 10 iterations. After obtaining the time until the next reaction tau, the next reaction executed after time tau is chosen according to the standard next-reaction Gillespie algorithm.

To identify the simulation time after which steady state is reached, simulation results with a time interval of 50 min are examined by eye. The timepoint after which no noticeable difference in the distributions is observed by eye was chosen as the simulation time. This simulation is generally the same between different simulations of neurites / dendrites / axons of the same length (e.g. for all simulations of 40 μm the same simulation time is used). Simulations of 30 μm neurites are generally run for 400 min, of 40 μm neurites and dendrites for 450 min, of 160 μm axons for 1600 min. The exact simulation times are noted for each panel in the figure legends.

Results for simulations of the MT-RF speedup experiment (in developing neurites and dendrites) are run until the steady state is reached, after which MT-RF speed is increased and data are extracted with a time interval of 1.5 min, which is the same interval as for experiments.

Results for simulations of MT decay experiments were extracted with a time interval of 0.5 min for up to 60 min (developing neurites and dendrites) or 90 min (dendrites and axons). The time interval matched experimental imaging times.

Mapping parameter values of growing-shrinking model to growing-pausing model

Switching of MTs between growing and shrinking^{27,34} is represented as a simplified switch between growing and pausing and the complete loss of the MT (catastrophe)

(Fig. 2B). To still fit our model to the distribution of growing MT tips and the total number of MTs at the base of the neurite, the ratio of the number of growing MT tips and the total number of MTs in our growing-pausing model needed to be the same as in the standard biophysical growing-shrinking model for MTs. In the growing-shrinking model this ratio is determined by the shrinkage rate (the transition from the growing to the shrinking state) and the rescue rate (the transition from the shrinking to the growing state). To obtain a realistic ratio of growing MTs to total MTs, we use the shrinkage rate of the growing-shrinking model as pausing rate in our growing-pausing model and keep the same rescue rate as for the growing-shrinking model.

MTs in the growing-shrinking model shrink in the shrinking state but do not change their length in our growing-pausing model. Thus, the growth velocity in our growing-pausing model needs to account for the shrinking in the growing-shrinking model by calculating an effective growth velocity v_g , which is lower than the actual MT growth velocity v_g . This effective growth velocity is calculated by matching the expected length of the growing-shrinking and growing-pausing model.

The growing-shrinking model is governed by the following two partial differential equations for the density of growing MT tips ρ_G and shrinking MT tips ρ_S :

$$\frac{\partial \rho_G(l)}{\partial t} = -\frac{\partial}{\partial l} v_g \rho_G + k_{\text{rescue}} \rho_S - k_{\text{shrink}} \rho_G, \quad F1$$

$$\frac{\partial \rho_S(l)}{\partial t} = \frac{\partial}{\partial l} v_s \rho_S - k_{\text{rescue}} \rho_S + k_{\text{shrink}} \rho_G. \quad F2$$

With v_g being the MT growth speed and v_s the MT shrinkage speed. The general steady state solution of ρ_G is:

$$\rho_G = C_1 * e^{-\left(\frac{k_{\text{shrink}}}{v_g} - \frac{k_{\text{rescue}}}{v_s}\right)l} + C_2. \quad F3$$

764 Thus, the expected length is:

$$765 \quad \langle l \rangle_{\text{growing-shrinking}} = \frac{1}{\frac{k_{\text{shrink}}}{v_g} - \frac{k_{\text{rescue}}}{v_s}}. \quad F4$$

766 The lifetime of MTs τ is approximately the time it takes for the average MT length $\langle l \rangle$
767 to depolymerize plus the time it takes to depolymerize the MT length l_τ growing during
768 the lifetime τ :

$$769 \quad \tau = \frac{\langle l \rangle + l_\tau}{t_s v_s}, \quad F5$$

$$770 \quad \tau = \frac{v_g \cdot v_s \cdot (k_{\text{rescue}} + k_{\text{shrink}})}{(-v_g k_{\text{shrink}} + v_s k_{\text{shrink}})(k_{\text{shrink}} v_s - k_{\text{rescue}} v_g)}. \quad F6$$

771 The MT length l_τ growing during the lifetime τ is calculated as:

$$772 \quad l_\tau = \tau \cdot t_g \cdot v_g, \quad F7$$

773 with t_s being the fraction of time a MT shrinks:

$$774 \quad t_s = \frac{\frac{1}{k_{\text{rescue}}}}{\frac{1}{k_{\text{rescue}}} + \frac{1}{k_{\text{shrink}}}}, \quad F8$$

775 and with t_g being the fraction of time a MT grows:

$$776 \quad t_g = \frac{\frac{1}{k_{\text{shrink}}}}{\frac{1}{k_{\text{rescue}}} + \frac{1}{k_{\text{shrink}}}}. \quad F9$$

777 To calculate the rescue rate k_{GS} , the following quadratic equation must be solved:

$$778 \quad 0 = k_{\text{rescue}}^2 + k_{\text{rescue}} \left(-\frac{2 \cdot v_s \cdot k_{\text{shrink}}}{v_g} - \frac{v_s}{\tau \cdot v_g} \right) - \frac{v_s}{\tau \cdot v_g} k_{\text{shrink}} + \frac{v_s^2}{v_g^2} k_{\text{shrink}}^2, \quad F10$$

$$k_{\text{rescue}} = \frac{v_s k_{\text{shrink}}}{v_g} + \frac{v_s}{2 \cdot \tau \cdot v_g} - \sqrt{\left(\frac{v_s k_{\text{shrink}}}{v_g} + \frac{v_s}{2 \cdot \tau \cdot v_g} \right)^2 + \frac{v_s}{\tau v_g} k_{\text{shrink}} - \frac{v_s^2}{v_g^2} k_{\text{shrink}}^2}. \quad F11$$

To solve this equation, we measured the shrinkage rate k_{shrink} from the average lifetime of growing MT tips in living neurons, visualized through fluorescently labeled end-binding protein 3 (EB3; Extended Data Fig. 2a-c)¹⁶. The calculated rescue rate k_{rescue} is used as the rescue rate for the growing pausing model. The measured shrinkage rate k_{shrink} is used as the pausing rate k_{pause} for the growing-pausing model:

$$k_{\text{pause}} = k_{\text{shrink}}. \quad F12$$

With the pausing rate k_{pause} and the rescue rate k_{rescue} set, the effective growth velocity v_{ge} of the growing-pausing model is calculated so that the expected length matches that of the growing-shrinking model. This effective growth velocity is lower than the actual MT growth speed to account for MTs shrinking.

The expected length of the growing-pausing model is:

$$\langle l \rangle_{\text{growing-pausing}} = \frac{v_{\text{ge}}}{\frac{k_{\text{pause}} k_{\text{rescue}}}{k_{\text{cat}} + k_{\text{rescue}} + k_{\text{stable}}} - k_{\text{pause}}}. \quad F13$$

Setting eq. F13 equal to F4 and solving for the effective growth velocity, v_d yields:

$$v_{\text{ge}} = \frac{k_{\text{pause}} k_{\text{rescue}}}{k_{\text{cat}} + k_{\text{rescue}} + k_{\text{stable}}} \cdot \frac{1}{\frac{k_{\text{shrink}}}{v_g} - \frac{k_{\text{rescue}}}{v_s}}. \quad F15$$

Fitting parameter values

To fit parameters, model results were compared to experimental data. Single or multiple parameters were varied to fit specific experimental data as described in Table

1. Parameter values were modulated manually, until the model results matched experimental data reasonably well.

Animals

All animal experiments were done in accordance with the Animal Welfare Act as well as the guidelines of the North Rhine-Westphalia State Environment Agency [Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV); reference number for approval (Aktenzeichen: 84-02.04.2015.A334 and Aktenzeichen 81-02.04.2021.A208)]. Animals were housed in groups (up to five mice per cage) with controlled room temperature at 21° to 22°C in addition to an artificial 12-hour light:12-hour dark cycle (lights off at 6:00 p.m.). Mice (adult C57Bl6/J) were given food and water ad libitum throughout the experiment.

Neuron culture

Primary hippocampal neurons were dissected from embryonic day 16.5 (E16.5) to E17.5 mouse brains. Following dissection, hippocampi were collected in Hanks' balanced salt solution with magnesium and calcium supplemented with 7 mM HEPES (Thermo Fisher Scientific, 14-025-050 and 15-630-056). Next, hippocampi were digested in 0.25% trypsin-EDTA supplemented with 7 mM HEPES (Thermo Fisher Scientific, 25-200-056) at 37°C for 15 min. Subsequently, they washed three times with minimum essential medium (MEM)–1% horse serum (MEM–1% HS) medium (1× MEM, 1× essential and nonessential amino acids, 2 mM L-glutamine (all from Thermo Fisher Scientific, 11-430-030, 11-130-036, 11-140-035, 25-030-081), 0.22% NaHCO₃, 0.6% glucose, and 1% HS (Biowest, S0910-500)). Finally, hippocampi were mechanically dissociated with fire-polished glass-Pasteur pipettes. Coverslips were coated with poly-L-Lysine (1 mg/ml) (Sigma-Aldrich, P2636) in borate buffer at room

temperature overnight, three to four times washed with double-distilled H₂O (ddH₂O) and placed in the incubator at 36.5°C and 5% CO₂ with MEM-HS 10% to equilibrate for at least 2 hours before plating. Neurons were cultured at 36.5°C and 5% CO₂ with a glia-feeding layer for staining neurons and without a glia-feeding layer for live-cell imaging, as previously described⁷⁶.

For fixating and staining neurons, 14 neurons/mm² were plated on 15 mm glass-coverslips with self-made paraffin dots. Two to four hours after plating, glass coverslips with neurons were flipped on glia feeding layer with neuronal medium (N2) (1x MEM, 1 mM sodium pyruvate (Sigma-Aldrich, S8636), 1% Neuropan 2 supplement (Pan-Biotech, P07-11100), NaHCO₃, 0.6% glucose, 2 mM L-glutamine and 1x B-27™ supplements (Thermo Fisher Scientific, 17-504-044) which was preconditioned for 3 days. For the glia feeding layer, 9 mouse glia cells/mm² were plated in a 6 cm culture dishes and kept in MEM-HS10%. Three days before culture of neurons, media was exchanged to N2 for preconditioning.

For live-cell imaging of neurons, after transfection, 2 * 10⁴ neurons were plated in eight-well glass-bottom dishes (ibidi, 80827-90). Two to three hours after plating, MEM-10% HS medium was replaced by neuronal medium (N2) (1xMEM, 1 mM sodium pyruvate, 1% Neuropan 2 supplement (Pan-Biotech, P07-11100), NaHCO₃, 0.6% glucose, 2 mM L-glutamine and 1xB-27™ supplements (Thermo Fisher Scientific, 17-504-044)), which was preconditioned by incubation on glia cells for two to three days.

Neuron transfections

Cell transfections were done using Nucleofector II (Lonza, AAB-1001) with program 0-005 for hippocampal mouse neurons. The mouse neuron Nucleofector Kit (Lonza,

VPG-1001) was used according to the manufacturer's instructions. For each transfection, 5×10^5 neurons were used with varying amounts for different plasmids: 15 or 25 μ g of pBetaActin-TUBB2a-mEos3.2 and 15 or 25 μ g of pBetaActin-TUBB2a-mEos3.2-mEos3.2-mEos3.2. All plasmids were purified using the EndoFree Maxiprep Kit (QIAGEN).

Live-cell imaging

All imaging experiments were performed on an Andor spinning disk Nikon Eclipse Ti microscope with Perfect Focus system (Nikon). The setup included a Yokogawa CSU-X1 spinning disk unit (CSUX1-A1N-E/FB2 5000 rpm Control, FW, DMB 95L100016) and a Plan Apo VC 60 $\times$ oil objective NA 1.4 with no additional magnifier controlled via the iQ3 software (Andor). Single band-pass emission filters for the blue channel (480/40; Semrock), the green channel (525/50; Semrock BrightLine), and the red channel (617/73; Semrock BrightLine) were used. Excitation of the green channel was done with a 488-nm laser (100 mW), for the red channel with a 561-nm laser (100 mW) (REVOLUTION 500 series AOTF laser modulator and combiner unit, solid-state laser modules). Imaging was conducted inside a temperature-controlled large incubator chamber around the microscope, kept at 37°C. A small chamber for eight-well ibidi glass-bottom dishes (Pecon, #000470) was supplied with humidified air mixed with CO₂ to maintain a concentration of 6–7%. Images were taken with an Andor iXON camera (DU897) in charge-coupled device mode at 1-MHz horizontal readout rate and with a pixel size of 0.22 μ m and no binning.

The growth cone for measuring MT shrinkage speed (Extended data Fig. 2f-g) was imaged with a pixel size of 39 nm, time interval of 5 s and using a SORA super resolution spinning disk on a Nikon confocal spinning disc microscope.

Images were taken every 30 s for the first 4 min, followed by every 2 min as specified in the “Photoconversion” section.

For all experiments, neurons were selected based on representative morphology of more than two neurites. Imaging was done using high light exposure for high fluorescence intensity, which was essential to assess MT stability by mEos.

Photoconversion

Photoconversion was performed using the fluorescence recovery after photobleaching and photoactivation (FRAPPA) illumination system (Andor, Revolution). The FRAPPA module directs the laser onto individual pixels for a defined dwell time, sequentially scanning through all pixels for a predetermined number of repetitions. A predefined 4-pixel wide area was selected for photoconversion in a minor neurite (DIV1) and the axon (DIV3) to minimize phototoxicity while achieving high conversion intensities. Two photoconversions were done in the same minor neurite and axon. One was close to the soma and one far away from the soma, on average at 30% and 68% of the neurite length and 12% and 35% of the axon length. The patches were converted consecutively (approx. 2 h apart), ensuring that no significant signal from the previous conversion was visible anymore for a similar baseline before each conversion. This approach ensured that both conversions did not influence each others intensity measurements.

Photoconversion was performed with the 405-nm laser (800 repetitions, 20 μ s dwell time, 1.78 μ W power, pixel size of 0.22 μ m, 10-pixel $\times$ 4-pixel). After photoconversion, images were acquired in the photoconverted (red) and not-photoconverted (green) channel every 30 s for the first 4.5 min, followed by every 2 min until most of the photoconverted mEos were depleted but at least 26 min in total. Exposure times were

set at 800 ms at 90% or 100% laser intensity to achieve an optimal signal to noise ratio. The same imaging settings and filters were maintained across all experiments to ensure comparable fluorescence intensity levels. Most cells were re-evaluated 1 to 3 h after photoconversion to ascertain cell survival post-experiment; cells that died were excluded from the analysis.

Photoconversion analysis of experiments for MT decay curve

To measure photoconverted intensity, first the background value was determined. The background was calculated in the entire neuron, except in the neurite containing the photoconverted spot. In that area the 50th highest pixel intensity value was measured for each timepoint and then the second highest value from all timepoints was increased by 2% and used as background value. Using this background value as threshold value to define the area with photoconverted intensity consistently worked well as judged by eye on most imaged neurons. This threshold value was subsequently subtracted from all intensity values and then the sum of all pixel intensity values in the neurite with the photoconverted spot was calculated for each timepoint. Once a noticeable part of the converted spot moved in the soma by eye, the analysis was stopped. This prevented photoconverted mass in the soma from affecting the analysis.

Photoconversion analysis of simulations for MT decay curve

To simulate the photoconversion experiment, for each MT the nucleation time, its x position, unstable length and stable length was saved. As before, simulations were first run until the steady state was reached and afterwards simulation data were saved each minute of simulation. After the simulation was fully done, the region where the photoconversion should be simulated was defined. At the first timepoint it was

recorded for each MT how much length is within the defined range, and all lengths were summed to obtain the starting MT mass. Then for each timepoint for every MT that was lost (identified by the time it was nucleated), the corresponding length in the range was subtracted from the starting MT mass. As for photoconversion experiments, new growth of MTs or newly nucleated MTs were not considered. This allowed obtaining a curve of the remaining MT mass over time and determining the MT decay curve.

MT decay curve analysis

Data were fitted with the dual exponential function:

$$MT_{\text{mass}}(t) = f_1 * e^{-\lambda_1 t} + f_2 * e^{-\lambda_2 t}, \quad G1$$

and for Fig. 1d also with the mono exponential function:

$$MT_{\text{mass}}(t) = f * e^{-\lambda t}, \quad G2$$

using `scipy.stats.curve`. For the dual exponential fit the fraction was calculated, e.g. for the fraction of population 1:

$$Fraction_1 = \frac{f_1}{f_1 + f_2}. \quad G3$$

Cells for which the two fitted rates λ_1 and λ_2 were less than three-fold different, only one population of MTs was considered to be present. In general, the faster and slower decay rate were assumed to represent unstable and stable MTs, respectively. However, if the decay rate was found to be smaller than 0.23 min^{-1} for developing neurites (corresponding to a half-life of ca. 3 min) or smaller than 0.1 min^{-1} for dendrites and axons, the corresponding population was assumed to indicate stable MTs. If the decay rate was larger than these threshold values, the population was assumed to

correspond to unstable MTs. For cases with only stable or only unstable populations present, an average decay rate was calculated:

$$\bar{\lambda} = Fraction_1 * \lambda_1 + Fraction_2 * \lambda_2. \quad G4$$

Measuring MT shrinkage speed

MT shrinkage speed was measured from single shrinkage events in one growth cone transfected with TUBB2a-mNeonGreen to be 10 $\mu\text{m}/\text{min} \pm 1 \mu\text{m}/\text{min}$ (n=5 events, N=1 growth cone; Extended data Fig. 2f-g). To account for MTs in the growth cone being pulled retrogradely due to actin retrograde flow, we reduced MT shrinkage speed by 3 $\mu\text{m}/\text{min}$, based on the measurement in³⁴.

Immunocytochemistry

Neurons were incubated for either 24 or 72 h at 36.5°C and 5% CO₂ and then fixed under conditions optimal for the preservation of the cytoskeleton: 4% paraformaldehyde, 4% sucrose in PHEM fixation buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 7.4) containing 0.1% Triton X-100 and 0.25% glutaraldehyde for 15 min. The coverslips with the cells were washed two times with PHEM buffer without fixatives and once with PBS. Fixed cells were quenched with 0.1 M glycine for 10 min, washed three times with PBS and then blocked at room temperature (RT) for 1 h with 2% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, A5256801), 2% Bovine Serum Albumin (BSA) (Sigma-Aldrich, A3294) and 0.2% fish gelatin (Sigma-Aldrich, G7765) in PBS. After blocking, cells were incubated with the primary antibodies (Tyrosinated Tubulin 1:800, Abcam ab6160; Detyrosinated Tubulin 1:500, Millipore MAB3201; Acetylated Tubulin 1:4000, Sigma T7451; Tubulin, alpha, (clone B-5-1-2) mouse monoclonal Ab, Sigma T5168, 1:4.000) for 1 h at RT. Cells were washed four times with PBS and incubated with the secondary antibodies (1:1000)

(Alexa-Fluor 488 α -Rat, Invitrogen, A21208; Alexa-Fluor 555 α -Rabbit, Invitrogen, A21429; Alexa-Fluor 555 α -Mouse, Invitrogen, A21422; Alexa-Fluor-488 anti-Mouse, Invitrogen, A11029), for 30 min at RT. The antibodies were mixed in a 1:10 dilution of the blocking solution in PBS. Cells were washed three times with PBS, once with ddH₂O and mounted onto microscope slides with Fluoromount™ (Sigma, F4680).

Imaging tubulin modifications in fixed cells

Cells for imaging of MT distribution were selected randomly. Cells were imaged using an Axio Observer.Z1 microscope equipped with a Colibri 7 LED light source and Zen Blue software (all from Zeiss). Images were taken with an Axiocam 512 mono camera using either a Plan Apochromat 63x oil objective numerical aperture (NA) 1.4 with a pixel size of 0.098 μ m (2 x 2 binning) or a Plan-Apochromat 40x oil objective NA 1.4 with a pixel size of 0.078 μ m (no binning). A dichroic Quad filter (QBS 405, 493, 575 and 653) and single band-pass emission filter for the green channel (525/50) and the red channel (605/40) were used. For the green and red channel, a 475 nm LED-module and a 555 nm LED-module were used.

Experimental MT distribution analysis

To analyze the experimental MT distribution, neurites were traced in FIJI. The traces were used in python to measure the summed intensity across the width of the neurite (up to a maximum of 31 pixels for the shaft and 71 pixels for growth cone) along the traced line. Images were background subtracted, with the background value identified for each cell separately as the highest value outside but close to the cell. For this a manual region close to the cell was drawn in FIJI and read out in python.

To compare MT decay data with experimental MT distributions (Fig. 1q and Extended Data Figs. 1e, 7i), a normalized ratio change, $ratio_{\text{Distribution}}^{\text{change}}$, was calculated for both

MT distributions and MT decay. This ratio change is invariant to the absolute ratio of stable to unstable MTs:

$$ratio_{MT\ decay}^{far} = \frac{fraction_{stable}^{far}}{fraction_{unstable}^{far}}, \quad H1$$

$$ratio_{MT\ decay}^{close} = \frac{fraction_{stable}^{close}}{fraction_{unstable}^{close}}, \quad H2$$

$$ratio_{MT\ decay}^{change} = \frac{ratio_{MT\ decay}^{far} - ratio_{MT\ decay}^{close}}{ratio_{MT\ decay}^{far} + ratio_{MT\ decay}^{close}}, \quad H3$$

$$ratio_{distribution}^{far} = \frac{Tubulin_{stable}^{far}}{Tubulin_{unstable}^{far}}, \quad H4$$

$$ratio_{distribution}^{close} = \frac{Tubulin_{stable}^{close}}{Tubulin_{unstable}^{close}}, \quad H5$$

$$ratio_{Distribution}^{change} = \frac{ratio_{distribution}^{far} - ratio_{distribution}^{close}}{ratio_{distribution}^{far} + ratio_{distribution}^{close}}. \quad H6$$

Calculating possible distributions for non-acetylated tubulin

Modifications of tubulin give an indication of the age of a MT – with acetylation and detyrosination accumulating on older MTs. Tyrosinated MTs should indicate young MTs and thus the distribution of tyrosinated MTs should be different from the distribution of acetylated and detyrosinated MTs. Instead, we found that the distribution for acetylated tubulin looked more similar to tyrosinated tubulin than to detyrosinated tubulin (Extended Data Fig. 1b). This indicates that the distribution of acetylated MTs contains a higher fraction of tyrosinated MTs than of detyrosinated MTs. Thus, the distribution of tyrosinated MTs also contains a substantial fraction of older MTs (stable MTs) that are acetylated. If the amount of MT modifications depends on the age of MTs, this would indicate that acetylation occurs noticeably faster than detyrosination – since

otherwise tyrosinated MTs would not be acetylated often. Indeed, in non-neuronal cells, MTs accumulate a lot of acetylation within 15 min after MT regrowth, but it takes more than 90 min to accumulate considerable fractions of detyrosination²⁶. This suggests that most MTs that are non-acetylated are tyrosinated and not detyrosinated, since they are not old enough to accumulate detyrosination. In summary, the distribution of non-acetylated should label MTs that are on average younger than tyrosinated MTs, since even some older MTs that are acetylated should still be tyrosinated. Thereby, non-acetylated tubulin could be a better marker for young, unstable MTs. To calculate possible distributions for non-acetylated MTs (that are also tyrosinated), average distributions for detyrosinated, tyrosinated and acetylated tubulin were used. Since acetylation/non-acetylation and detyrosination/tyrosination can occur together but MTs cannot be acetylated and non-acetylated or detyrosinated and tyrosinated at the same time, the following four combinations of modifications are possible for MTs: acetylated and tyrosinated, acetylated and detyrosinated, non-acetylated and tyrosinated as well as non-acetylated and detyrosinated. We measured the following three distributions experimentally:

$$MT_{\text{acet}}^{\text{exp}}(x) = \frac{MT_{\text{acet-detyr}} + MT_{\text{acet-tyr}}}{MT_{\text{acet-detyr}}(0) + MT_{\text{acet-tyr}}(0)}, \quad I1$$

$$MT_{\text{tyr}}^{\text{exp}}(x) = \frac{MT_{\text{acet-tyr}} + MT_{\text{nonacet-tyr}}}{MT_{\text{acet-tyr}}(0) + MT_{\text{nonacet-tyr}}(0)}, \quad I2$$

$$MT_{\text{detyr}}^{\text{exp}}(x) = \frac{MT_{\text{acet-detyr}} + MT_{\text{nonacet-detyr}}}{MT_{\text{acet-detyr}}(0) + MT_{\text{nonacet-detyr}}(0)}. \quad I3$$

Since the fluorescent signals from the three markers are arbitrary units, we normalized each distribution by the value in the neurite close to the soma ($x=0$). Therefore, the experimental measurements only allow us to obtain a relative distribution and leaves

the ratio of the three distributions unknown – e.g. we cannot measure how much more acetylation there is compared to detyrosination.

Since detyrosination happens a lot slower than acetylation in dividing cells²⁶, we don't expect MTs that are detyrosinated to still be nonacetylated. Thus, we assumed that the ratio of MTs that are non-acetylated and detyrosinated to MTs that are acetylated and detyrosinated is close to 0:

$$ratio_{\frac{\text{acet-detyr}}{\text{nonacet-detyr}}} = \frac{MT_{\text{acet-detyr}}}{MT_{\text{nonacet-detyr}}} \approx 0. \quad I4$$

Thus, the measured distribution of detyrosinated MTs should represent the distribution of detyrosinated MTs that are also acetylated:

$$MT_{\text{detyr}}^{\text{exp}}(x) = \frac{MT_{\text{acet-detyr}}}{MT_{\text{acet-detyr}}(0)}. \quad I5$$

We now assume different fractions of acetylated MTs that are also detyrosinated (0.05, 0.1 and 0.15) in the neurite close to the soma (x=0):

$$fraction_{\frac{\text{acet-detyr}}{\text{acet-tyr+acet-detyr}}} = \frac{MT_{\text{acet-detyr}}(0)}{MT_{\text{acet-tyr}}(0) + MT_{\text{acet-detyr}}(0)}, \quad I6$$

and different fractions of tyrosinated MTs that are also acetylated (5%, 15%, 25%, 35%, 45%, 55%, 65% and 75%) in the neurite close to the soma (x=0):

$$fraction_{\frac{\text{acet-tyr}}{\text{acet-tyr+nonacet-tyr}}} = \frac{MT_{\text{acet-tyr}}(0)}{MT_{\text{acet-tyr}}(0) + MT_{\text{nonacet-tyr}}(0)}. \quad I7$$

We combine equations (I5), (I7) and (I1) to use the $fraction_{\frac{\text{acet-detyr}}{\text{acet-tyr+acet-detyr}}}$ for calculating the distribution of MTs that are acetylated and tyrosinated:

$$MT_{\text{acet-tyr}}^{\text{norm}}(x) = MT_{\text{acet}}^{\text{exp}} - fraction_{\frac{\text{acet-detyr}}{\text{acet-tyr+acet-detyr}}} MT_{\text{detyr}}^{\text{exp}}, \quad I8$$

$$MT_{\text{acet-tyr}}^{\text{norm}}(x) = \frac{MT_{\text{acet-detyr}} + MT_{\text{acet-tyr}}}{MT_{\text{acet-detyr}}(0) + MT_{\text{acet-tyr}}(0)} - \frac{MT_{\text{acet-detyr}}(0)}{MT_{\text{acet-tyr}}(0) + MT_{\text{acet-detyr}}(0)} \frac{MT_{\text{acet-detyr}}}{MT_{\text{acet-detyr}}(0)}, \quad I9$$

$$MT_{\text{acet-tyr}}^{\text{calc}}(x) = \frac{MT_{\text{acet-tyr}}}{MT_{\text{acet-detyr}}(0) + MT_{\text{acet-tyr}}(0)}. \quad I10$$

We normalize equation (I10) by its value in the neurite close to the soma, to obtain the normalized distribution of MTs that are acetylated and tyrosinated:

$$MT_{\text{acet-tyr}}^{\text{norm}}(x) = \frac{MT_{\text{acet-tyr}}^{\text{calc}}}{MT_{\text{acet-tyr}}^{\text{calc}}(0)} = \frac{MT_{\text{acet-tyr}}}{MT_{\text{acet-tyr}}(0)}. \quad I11$$

Finally, we combine equations (I2), (I7) and (I10) to subtract this distribution from the distribution of tyrosinated MTs, resulting in normalized distribution of non-acetylated MTs that are tyrosinated, which is the distribution of non-acetylated MTs we used as marker for unstable MTs:

$$MT_{\text{nonacet-tyr}}^{\text{calc}}(x) = MT_{\text{tyr}}^{\text{exp}} - \text{fraction}_{\text{acet-tyr+nonacet-tyr}} MT_{\text{acet-tyr}}^{\text{norm}}, \quad I12$$

$$MT_{\text{nonacet-tyr}}^{\text{calc}}(x) = \frac{MT_{\text{acet-tyr}} + MT_{\text{nonacet-tyr}}}{MT_{\text{acet-tyr}}(0) + MT_{\text{nonacet-tyr}}(0)} - \frac{MT_{\text{acet-tyr}}(0)}{MT_{\text{acet-tyr}}(0) + MT_{\text{nonacet-tyr}}(0)} \frac{MT_{\text{acet-tyr}}}{MT_{\text{acet-tyr}}(0)}, \quad I13$$

$$MT_{\text{nonacet-tyr}}^{\text{calc}}(x) = \frac{MT_{\text{nonacet-tyr}}}{MT_{\text{acet-tyr}}(0) + MT_{\text{nonacet-tyr}}(0)}. \quad I14$$

Lastly, by normalizing the distribution by its value in the neurite close to the soma (x=0), we obtain the normalized distribution of nonacetylated MTs that are tyrosinated:

$$MT_{\text{nonacet-tyr}}^{\text{norm}}(x) = \frac{MT_{\text{nonacet-tyr}}^{\text{calc}}}{MT_{\text{nonacet-tyr}}^{\text{calc}}(0)} = \frac{MT_{\text{nonacet-tyr}}}{MT_{\text{nonacet-tyr}}(0)}. \quad I15$$

1064 Measuring the number of MTs at the neurite base

1065 Estimating the number of MTs at the neurite base was done similarly as in³⁴. The
 1066 measured total intensity of alpha Tubulin was divided by the intensity of a single MT,
 1067 to obtain an estimate for the number of MTs (Extended Data Fig. 2d,e). Single MTs
 1068 could not be identified visually in the neurite or soma, but in the growth cone. For these
 1069 single MTs, the intensity of one pixel length was measured across the entire MT. All
 1070 intensity values for one pixel length MT from one cell were averaged. The intensity of
 1071 alpha Tubulin at the neurite base was then normalized by this average single MT
 1072 intensity per pixel length.

1073 Analysis of EB3 lifetime and distribution

1074 Live-cell imaging movies of EB3 were processed automatically with the
 1075 “plusTipTracker” of “u-track 2.0” implemented in Matlab⁷⁷. For detecting comets, a
 1076 “low-Gaussian SD” of 1 pixel, “high-pass Gaussian SD” of 4 pixels, and a “minimum
 1077 threshold” of 3 SDs with a “threshold step size” of 1 SD were employed. To track
 1078 comets, these parameters were used: “maximum gap to close” was 2 frames,
 1079 “minimum length of TrackSegments from First Step” was 3 frames, with segments
 1080 being split. For “Cost functions” and “Kalman filter functions” the option “Microtubule
 1081 plus-end dynamics” was used. To obtain the distribution of EB3 comets, further
 1082 analysis of comet tracks was performed using the previously developed Python
 1083 package “neuriteMT”¹⁶ (<https://github.com/maxschelski/neuritemt>). This package
 1084 builds an image of the neuron using all comet tracks identified from all timepoints. This
 1085 neuron image was then processed further using the pyNeurite package¹⁶, which
 1086 created sorted skeleton points of all neurites. For each timepoint a distribution of the

number of EB3 comets per μm was obtained for all neurites. EB3 comets attributed to the first pixel of the neurite were excluded to remove EB3 comets from the soma. These distributions were averaged across all neurites from 20 μm to 40 μm long to obtain the average distribution of EB3 comets in the neurite.

To obtain the lifetime of EB3 comets (Extended data Fig. 2a-c), we calculated the histogram of the measured EB3 lifetimes (seconds they were present) and fitted a mono-exponential function to it using the `curve_fit` function in the python package `scipy.optimize`:

$$N_{\text{EB3}} = x(0) * e^{-\lambda x}. \quad J1$$

Where N_{EB3} is the number of EB3 comets with a specific lifetime (in the histogram bin) and x is the measured lifetime of EB3 comets. The fitted lifetime $1/\lambda$ was used for the analysis. Histograms were obtained for each neuron and the average of the lifetimes of all cells was used.

Analysis of MT loss for MT-RF speedup experiments

First, the distribution of MTs was analyzed, as defined in “Experimental MT distribution analysis”. Then either the average intensity in the entire neurite (global) or the intensity from 60% to 90% neurite length towards the neurite tip (distal) was analyzed for each timepoint.

For neurites, tracing was done once for all timepoints, to analyze local MT mass in the neurite for each timepoint. The first timepoint after the treatment started (clearly before MT-RF speedup) was chosen as the starting value. The second timepoint was separately chosen for global and distal MT mass as the lowest value within 15 min

1109 after treatment started. Timepoints for the analysis of simulations were chosen the
1110 same way.

1111 For dendrites, due to massive changes in neurite shape, local dendritic MT mass was
1112 only analyzed at two timepoints, by tracing dendrites separately at these two
1113 timepoints. The baseline timepoint was the first timepoint after the treatment had
1114 started, as for neurites. To measure MT loss, the second timepoint was chosen by eye
1115 to be within 15 min after the timepoint with the fastest visible MT-RF speed. Due to
1116 variable onset times of visible MT-RF speedup and variable MT-RF acceleration, the
1117 corresponding timepoints varied between 12 and 144 min. For simulation data, to
1118 match the experimental timeframe, the timepoint with the lowest number of MTs either
1119 globally or distally within 60 min after MT-RF speedup was chosen. This way, the
1120 average timeframe used for the analysis of simulation data for the entire neurite
1121 (global) was 51 min after speedup, exactly like for experimental data.

1122 Python scripts

1123 All scripts used in the manuscript were written with Python 3.7 or 3.8. Environments
1124 were constructed with conda or mamba using the conda-forge channel (Anaconda
1125 Inc., 2020; available from <https://docs.anaconda.com/>; [https://github.com/mamba-](https://github.com/mamba-org/mamba)
1126 [org/mamba](https://github.com/mamba-org/mamba)). The following packages were used: SciPy 1.6.2 (for FigureFlow) or 1.5.2
1127 (<https://pypi.org/project/scipy/>)⁷⁸, sci-kit image 0.17.2 or 0.18.1
1128 (<https://pypi.org/project/scikit-image/>)⁷⁹, numpy 1.20.3
1129 (<https://pypi.org/project/numpy/>)⁸⁰, seaborn 0.11.1 (for FigureFlow) or 0.11.0
1130 (<https://pypi.org/project/seaborn/>)⁸¹, scikit-posthocs 0.6.7
1131 (<https://pypi.org/project/scikit-posthocs/>)⁸², matplotlib 3.34 or 3.4.2
1132 (<https://pypi.org/project/matplotlib/>)⁸³, pandas 1.3.0 (for FigureFlow) or 1.1.3

1133 (<https://pandas.pydata.org/>)⁸⁴, python-pptx 0.6.19 ([https://pypi.org/project/python-](https://pypi.org/project/python-pptx/)
1134 [pptx/](https://pypi.org/project/python-pptx/)). For cytostoch the following packages were used: numba 0.60
1135 (<https://github.com/numba/numba>), pytorch 2.2.2 (<https://pytorch.org/>), pandas 2.1.1
1136 (<https://pandas.pydata.org/>)⁸⁴, pyarrow 11.0 (<https://pypi.org/project/pyarrow/>),
1137 matplotlib 3.8.0 (<https://pypi.org/project/matplotlib/>)⁸³, seaborn 0.12.2
1138 (<https://pypi.org/project/seaborn/>)⁸¹, dill 0.3.7 (<https://pypi.org/project/dill/>).

1139 **Figure preparation (FigureFlow)**

1140 All figures were prepared using our Python package FigureFlow¹⁶. The package is
1141 available via GitHub (<https://github.com/maxschelski/figureflow>).

1142 **Statistics**

1143 Generally, only statistical comparisons between experimental data are shown. We
1144 decided not to perform statistical comparisons between simulation data and
1145 experimental data for two reasons. First, we do not want to claim that experimental
1146 data and simulation data are identical. Instead, we want to highlight that our
1147 simulations often capture the main features of experimental data and usually are
1148 quantitatively close to experimental data. Second, the large number of simulations for
1149 model data can make even very small differences significant. This can lead to
1150 significant differences, when distributions by eye seem to not differ a lot. Instead of
1151 statistical comparisons, for all data, except distributions across the neurite, we show
1152 boxplots of all simulation data and experimental data to allow a good comparison
1153 between model and experimental data by eye. This allows to judge not only how big
1154 the difference of the averages is but also how much of the variance in experimental
1155 data is captured by the stochastic variations in the model. For all data where more
1156 than two groups were compared, the nonparametric Kruskal-Wallis test

1157 (scipy.stats.kruskal_wallis) was first performed. If the resultant P value was below
1158 0.05, all combinations of groups were compared. All statistical comparisons of the two
1159 groups were undertaken using the nonparametric Dunn's test (scikit-
1160 posthocs.posthoc_dunn), with Bonferroni correction. If only two groups were
1161 compared, the Mann-Whitney U test was used.

1162 **Data availability**

1163 Previously acquired experimental data that was analyzed in this study is publicly
1164 available (<http://doi.org/10.5061/dryad.2fqz612s8>). Experimental data newly acquired
1165 for this study and model data will be made publicly available.

1166

1167 **Code availability**

1168 The Python package to run stochastic simulations of MTs in neurites developed for
1169 this study is available from Github (<https://github.com/maxschelski/cytostoch>). Further
1170 Python packages used in this study are publicly deposited
1171 (<http://doi.org/10.5281/zenodo.6990848>). All scripts used in this study to generate
1172 figures and data will be publicly deposited.

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Author Information

Contributions

M.S. conceived the project. M.S., N.P. and F.B. designed the research. M.S. and N.P. developed the theory. M.S., T.P., C.G. and S.S. performed the experiments. M.S. and C.G. performed analysis of raw images. M.S. analyzed the data. M.S. developed the software. M.S. N.P. and F.B. supervised the research. M.S., N.P. and F.B. wrote the manuscript. C.G., T.P. and S.S. reviewed the manuscript.

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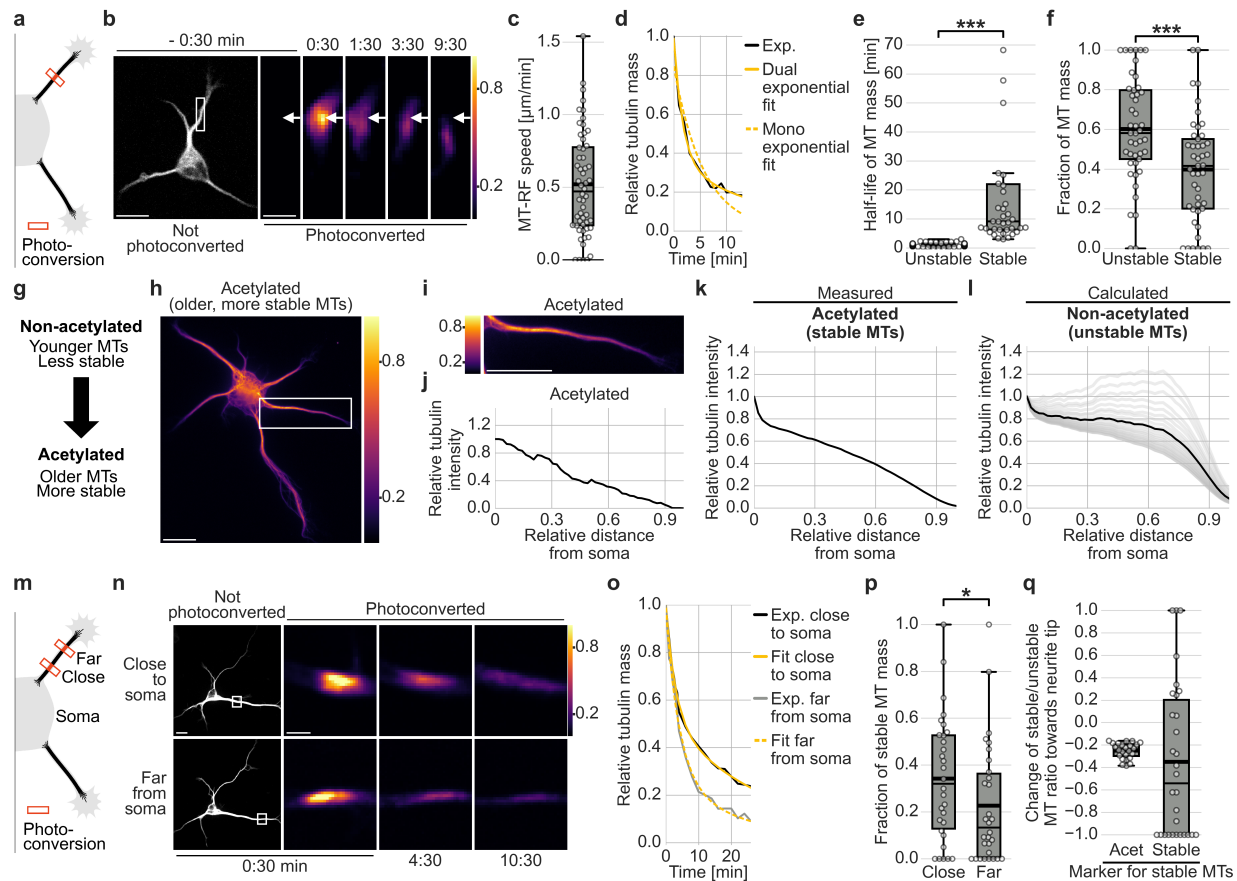


Fig. 1: Stable and unstable MTs are differently distributed in developing neurites.

a-f, The tubulin TUBB2a was fused to the photoconvertible fluorophore mEos3.2 and expressed in neurons in which a small MT patch was photoconverted after one day in culture. **(a)** Illustration of photoconversion experiment for **b-f**. **(b)** Representative images of the not photoconverted channel and the photoconverted channel from before to after photoconversion for **c-f**. The white arrow marks the position of the photoconverted MT array at the start. Time is in min:seconds. Scale bars, 10 μm for not zoomed and 2 μm for zoomed images. **(c)** MT-RF was measured as the distance that the photoconverted MT patch moved per time (n = 48 neurons, N = 4 experiments). **(d-f)** The photoconverted fluorescent intensity in the entire neurite was summed at each timepoint. **(d)** The resulting decay curve (Exp.) was then fitted to either a mono- or dual-exponential function, which indicates one population or two populations of decaying MTs. **(e,f)** **(e)** Half-life and **(f)** fraction of the photoconverted MT mass of the fast-decaying component (unstable MTs) and the slow decaying component (stable MTs) of the dual exponential fit were plotted. For stable MT half-life in **e** three datapoints with a half-life above 10,000 min are out of the plotting range. **(e:** n = 38 neurons for stable and n = 43 neurons for unstable MTs, N = 4, p = 5.5 * 10⁻¹⁵; **f:** n = 45 neurons for both groups, N = 4, p=0.0003; Mann-Whitney U test). **g-l,** Neurons were stained after one day in culture for tyrosination as a marker for younger and detyrosination as well as acetylation as a marker for older MTs. Intensities were quantified for each point along the neurite as the sum of all pixel intensities across the width of the neurite. Tubulin intensity was normalized to the value closest to the soma. **(g)** Illustration of MT acetylation indicating older, more stable MTs. **(h-j)** Representative image **(h)** of the entire neuron and **(i)** the region marked with the white rectangle enlarged, and **(j)** the intensity curve along the neurite from **i**, stained for acetylated tubulin. Scale bars, 10 μm. **(k)** Distributions of tubulin modifications were averaged for all

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neurites (n = 505 neurites from 143 neurons, N = 3 experiments). **(l)** Based on average distributions of tyrosinated, detyrosinated and acetylated tubulin as well as assumptions about the fraction of co-occurrence of MT modifications, several possible distributions of non-acetylated tubulin were calculated (grey). The average of the possible distributions for non-acetylated tubulin is plotted in black (Methods section “Calculating non-acetylated tubulin distribution”). **m-q**, In neurons expressing TUBB2a fused to mEos3.2, a small area of MTs was photoconverted close to or far from the soma, at an average 30% and 68% neurite length towards the neurite tip, respectively. The photoconverted intensity was summed for each timepoint and the decay curve fitted to a dual exponential function. **(m)** Illustration of photoconversion experiment. **(n)** Images of photoconverted tubulin of a representative neuron for **p**. Time is in min:seconds. Scale bars, 10 μ m for not zoomed and 2 μ m for zoomed images. **(o)** Decay curves of the representative neuron in **n**, one photoconverted close to and one far from the soma (Exp.) with the corresponding dual exponential fits. **(p)** The fraction of stable MT mass close to and far from the soma (n = 30 neurons, N = 3 experiments; p = 0.029, Mann-Whitney U test). **(q)** The normalized change of the ratio of stable to unstable MTs from close to the soma to far from the soma was calculated and normalized by the sum of the ratios at both positions. Acetylated and non-acetylated tubulin were used as markers for stable and unstable MTs, respectively (Acet). This was compared to the value obtained from the data in **p** as a good marker for stable MTs (Stable). Intensity in **b**, **h**, **i** and **n** is color-coded from purple (low) to yellow (high). The thick line in boxplots indicates the mean, the thin line the median.

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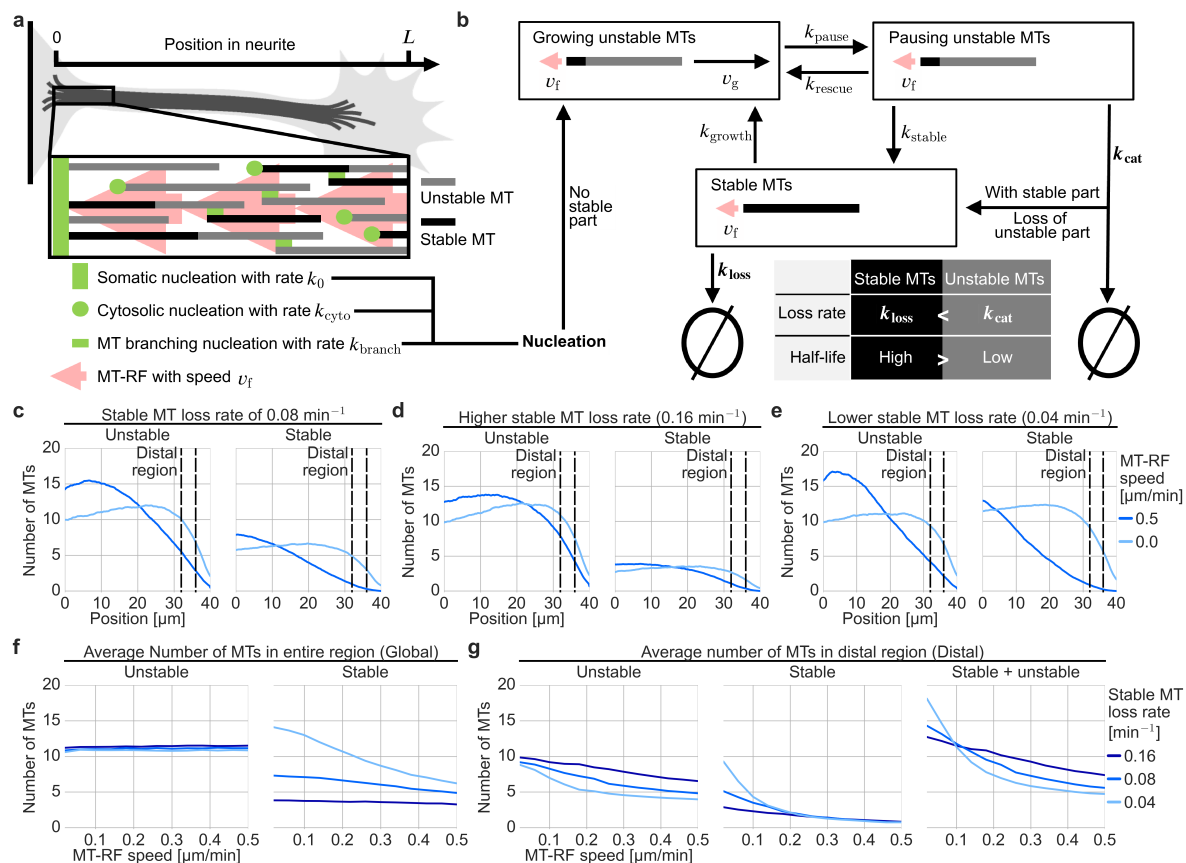


Fig. 2: MT-RF reduces stable and distal MTs depending on the stable MT loss rate.

a, Illustration of the three mechanisms for MT nucleation and MT-RF. **b**, Illustration of the states that MTs can be in, the transitions between these states and how new MTs can be generated and MTs can be lost through catastrophe. The illustration also shows the key difference of stable and unstable MTs in their loss rate and half-life. **c-g**, Simulations with different MT-RF speeds (0 – 0.5 $\mu\text{m/min}$) and different stable MT loss rates (0.04, 0.08, 0.16 min^{-1}) ($n = 1000$ simulations per condition, simulation time: 450 min). (**c-e**) The distribution of unstable and stable MTs is plotted for simulations with fast MT-RF speed of 0.5 $\mu\text{m/min}$ or without MT-RF (MT-RF speed of 0 $\mu\text{m/min}$) for different stable MT loss rates. Dashed lines indicate the region used to calculate distal MTs for the analysis in **g**. (**f-g**) The number of MTs averaged (**f**) in the entire region (global) or (**g**) from 80% (32 μm) to 90% (36 μm) region length towards the end of the region (distal) when changing the MT-RF speed for different stable MT loss rates.

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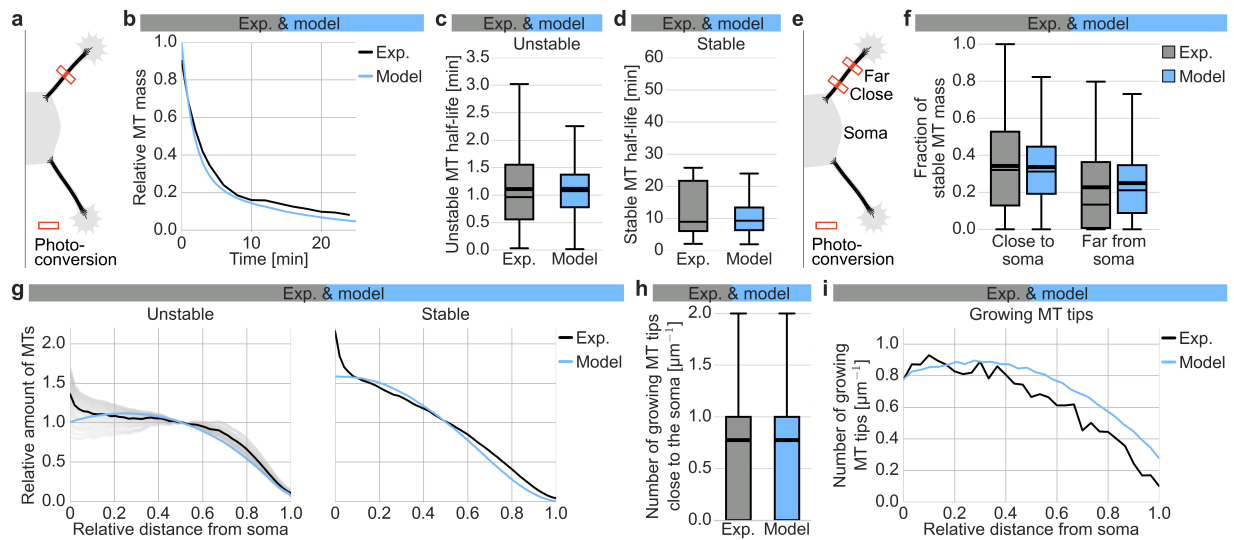


Fig. 3: The model reproduces experimental data in developing neurites.

a-d, Simulations of the photoconversion experiment over 40 μm are run and results compared to experimental data from Fig. 1, d-f. The total photoconverted MT mass was measured at all timepoints and the decay curve fitted to a dual exponential function. **(a)** Illustration of photoconversion experiment (model for b-d: 1000 simulations, simulation time: 510 min). **(b)** Average MT mass decay curve ($n = 43$ neurons, $N = 4$ experiments). **(c-d)** The half-life of **(c)** the fast-decaying component (unstable MTs) or **(d)** the slow-decaying component (stable MTs) (Exp.: **c**: $n = 43$ neurons, $N = 4$ experiments; **d**: $n = 40$ neurons, $N = 4$ experiments). **e-f**, Simulations of photoconversion close to and far from the soma over 40 μm are run and results are compared to experimental data from Fig. 1p. **(e)** Illustration of photoconversion experiment. **(f)** The fraction of stable MT mass close to and far from the soma was calculated from the dual exponential fit of the slow and fast decaying component ($n = 1000$ simulations, simulation time: 510 min). **g**, Distribution of stable and unstable MTs for simulations over 30 μm and experiments from Fig. 1k-l with an average neurite length of 30 μm . For simulation data, the distribution of stable MTs and unstable MTs is summed for all MT populations. Values are normalized to the value in the middle of the neurite (Exp: Stable (acetylated): $n = 505$ neurites from 143 neurons, $N = 3$ experiments; Model: $n = 16000$ simulations, simulation time: 450 min). **h,i**, The density of growing MT tips was calculated from the data of EB3 experiments from ¹⁶ with an average neurite length of 30 μm . The density of growing MT tips is calculated from simulations over 30 μm ($n = 37$ neurons, $N = 3$ experiments; **g-i**: $n = 16000$ simulations, simulation time: 450 min). The density **(h)** close to the soma or **(i)** across the entire neurite was plotted. Due to the large number of simulations, model and experimental data are not statistically compared (Methods section “Statistics”). The thick line in boxplots indicates the mean, the thin line the median.

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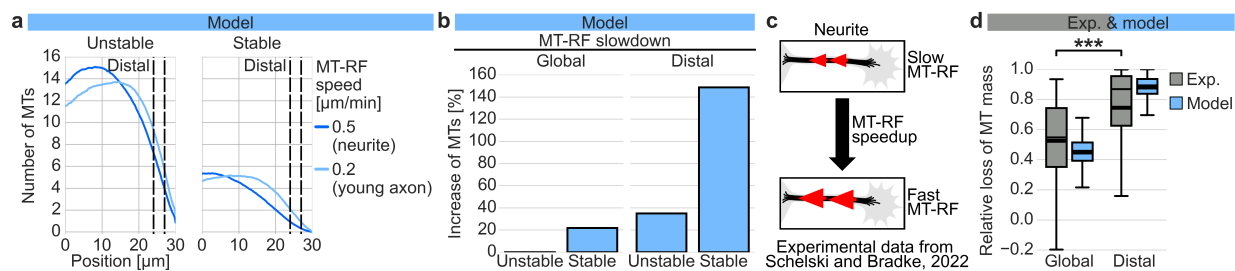


Fig. 4: MT-RF slowdown in young axons increases stable MTs in the distal neurite.

a,b, Simulations over 40 μm are run with the fast MT-RF speed of developing neurites (0.5 μm/min) or the slow MT-RF speed of young, 1-day old axons (0.2 μm/min; $n = 2000$ simulations, simulation time: 450 min). **(a)** The distributions of stable and unstable MTs summed from all populations are quantified with the region used to calculate distal MTs in **b** marked by dashed lines. **(b)** The average number of stable and unstable MTs in the entire neurite (global) or from 32 μm (80%) to 36 μm (90%; distal) is calculated. The increase in MTs for slow MT-RF of 0.2 μm/min compared to fast MT-RF of 0.5 μm/min is calculated. **c,d**, Analyzed experimental raw data and simulations of the MT-RF speedup experiment from ¹⁶ (raw data underlying Fig. 10, A to F). **(c)** Illustration of MT-RF speedup experiment in **d**. **(d)** The loss of MTs was calculated as the difference of the MT mass before MT-RF speedup to the lowest MT mass up to 15 min after the start of MT-RF speedup. MT mass loss was calculated in the entire neurite (global) or from 60% to 90% neurite length towards the neurite tip. Simulations are run over 32 μm, the average length of a neurite in the experimental data. For simulations, MT-RF speed was increased from 0.2 μm/min to 2.41 μm/min after 450 min (when the steady state was reached), with these values being the experimentally measured average MT-RF speeds before and after speedup (Experiment: $n = 59$ neurites from 21 cells, $N = 4$ experiments; Simulation: $n = 500$, simulation time: 549 min; $p = 3.8 \times 10^{-7}$, Dunn's test. Due to the large number of simulations, model and experimental data are not statistically compared and thus only statistically significant differences between experimental data are shown (Methods section "Statistics"). The thick line in boxplots indicates the mean, the thin line the median.

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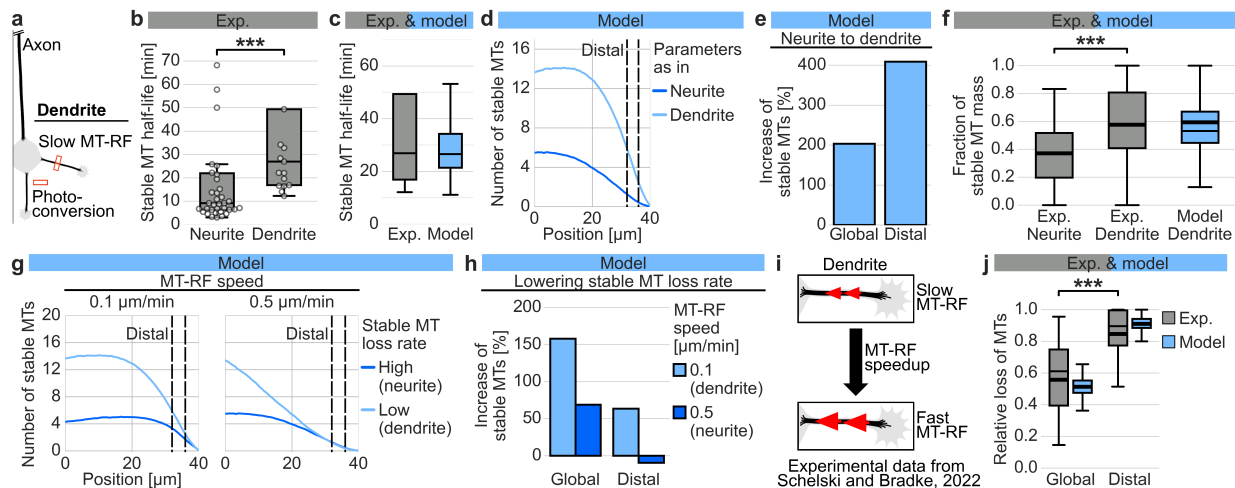


Fig. 5: MT-RF slowdown in dendrites increases stable MTs globally and distally.

a,b, Neurons cultured for six to seven days and expressing the tubulin TUBB2a fused to the photoconvertible fluorophore mEos3.2 were imaged after a small MT patch was photoconverted in dendrites, at an average 46% neurite length towards the neurite tip (Raw data from Fig. S7¹⁶). The photoconverted MT mass was measured at each timepoint and the decay fitted to a dual exponential function. **(a)** Illustration of the photoconversion experiment in dendrites and slow MT-RF as the main parameter change used to simulate dendrites. **(b)** The half-life of stable MTs was calculated from the fitted rate of the slow decaying component. For neurites and dendrites, three and four datapoints with a half-life above 500 min are out of the plotting range, respectively. (Dendrite: $n = 17$ neurons, $N = 1$ experiment; Data from neurites from Fig. 1e: $n = 38$ neurons, $N = 4$ experiments; $p = 0.0004$, Mann-Whitney U test) **c**, Simulations with the measured slow dendritic MT-RF speed of $0.1 \mu\text{m}/\text{min}$ were run and the stable MT loss rate was reduced (from 0.15 min^{-1} to 0.05 min^{-1}) to obtain the experimental stable MT half-life from **b** ($n = 1000$ simulations, simulation time: 540 min). **d,e**, Simulations over $40 \mu\text{m}$ are run with the fast fitted stable MT loss rate (Fig. 3d) and fast MT-RF speed of $0.5 \mu\text{m}/\text{min}$ from developing neurites (Neurite) or the slow fitted stable MT loss rate (fitted in **c**) and slow MT-RF speed of $0.1 \mu\text{m}/\text{min}$ from dendrites (Dendrite) ($n = 2000$ simulations, simulation time: 450 min). **(d)** The distribution of stable MTs is plotted with the region used to calculate distal MTs in **(e)** marked by dashed lines. **(e)** The average number of MTs in the entire region (global) or from $32 \mu\text{m}$ (80%) to $36 \mu\text{m}$ (90%; distal) is calculated. The increase in stable MTs for parameter values simulating the dendrite compared to parameter values simulating the neurite is calculated ($n = 500$ simulations, simulation time: 350 min). **f**, The fraction of stable MT mass from experimental data of neurites at 30% neurite length towards the neurite tip (Fig. 1f; Exp neurite) and of dendrites at 46% dendrite length towards the neurite tip (Exp. Dendrite) were compared to simulations with parameter values corresponding to experimental data from dendrites (Exp. neurite data from Fig. 1f: $n = 45$ neurons, $N = 4$ experiments; Exp dendrite: $n = 18$ neurons, $N = 1$ experiment; $p = 9.4 \cdot 10^{-5}$, Dunn's test; Simulation: $n = 1000$ simulations, simulation time: 540 min). **g,h**, Simulations over $40 \mu\text{m}$ are run with combinations of dendritic slow MT-RF ($0.1 \mu\text{m}/\text{min}$) and fast MT-RF of developing neurites ($0.5 \mu\text{m}/\text{min}$) with the high (0.15 min^{-1}) and low (0.05 min^{-1}) stable MT loss rate in developing neurites and dendrites, respectively ($n = 2000$ simulations, simulation time: 450 min). **(g)** Stable MT distributions are shown with the region used to calculate distal MTs in **h** marked by dashed lines. **(h)** MTs in the entire region (global) or from $32 \mu\text{m}$ (80%) to $36 \mu\text{m}$ (90%; distal) are analyzed. Then the increase in stable MTs for slower stable MT loss rate (0.05 min^{-1}) compared to fast stable MT loss rate (0.15 min^{-1}) is calculated. **i,j**, Simulations and analyzed raw experimental data of the MT-RF speedup experiment in dendrites from ¹⁶ (raw

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1523 data underlying Fig. S12). (i) Illustration of speedup experiment. (j) Raw experimental data
 1524 was from ¹⁶ and simulation data is analyzed right before dimerization and on average 50 min
 1525 to 60 min after MT-RF speedup, either in the entire neurite (global) and from 60% to 90%
 1526 neurite length towards the neurite tip (distal). Simulations were run over 42 μm , the average
 1527 dendrite length in experimental data. For simulations, MT-RF speed was increased from 0.05
 1528 $\mu\text{m}/\text{min}$ to 1.32 $\mu\text{m}/\text{min}$ after 450 min (when the steady state was reached), these values being
 1529 the experimentally measured average MT-RF speeds before and after speedup (raw data
 1530 underlying ¹⁶, Fig. S12; Exp: $n = 17$ neurons, $N = 3$ experiments; Sim: $n = 500$ simulations,
 1531 simulation time: 570 min; $p = 0.00097$, Dunn's test). The thick line in boxplots indicates the
 1532 mean, the thin line the median. Due to the large number of simulations, model and
 1533 experimental data are not statistically compared and thus only statistically significant
 1534 differences between experimental data are shown (Methods section "Statistics").

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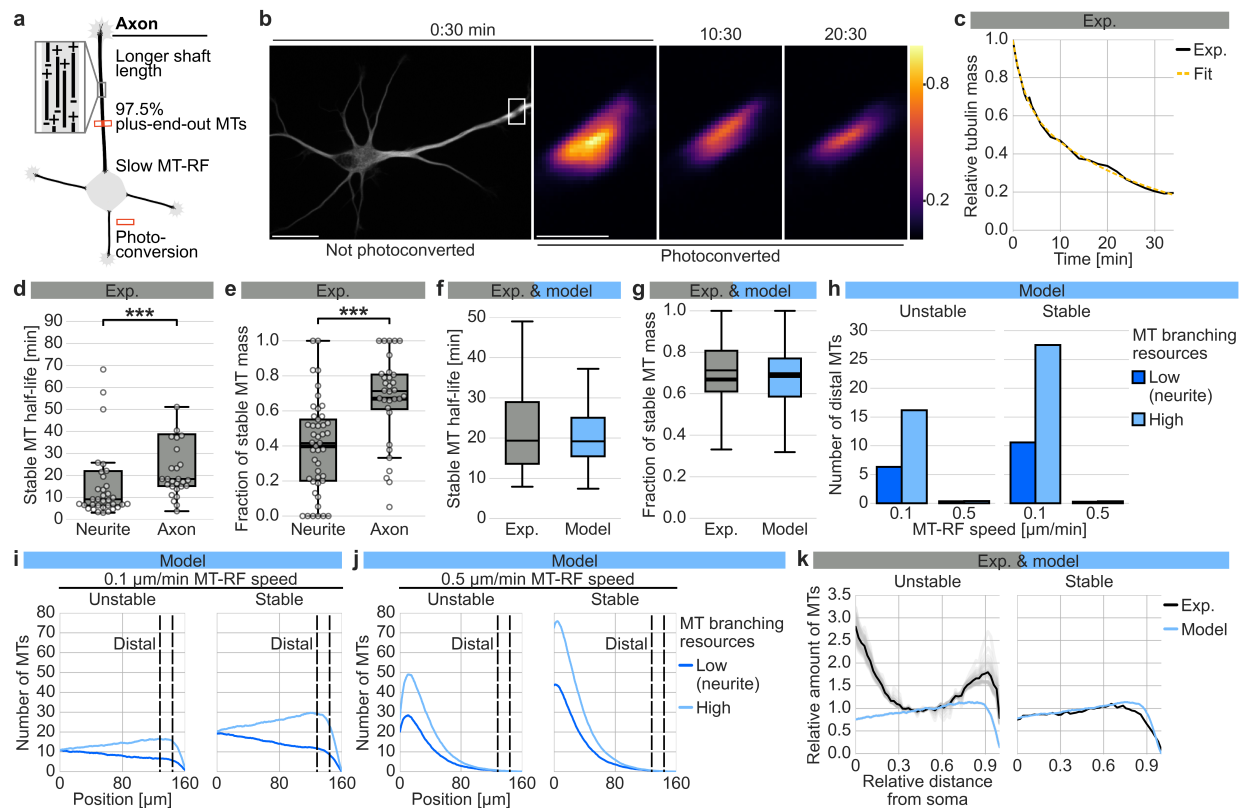


Fig. 6: MT-RF slowdown in axons enables stable MTs to increase towards the axon tip

a-e, Neurons expressing the tubulin TUBB2a fused to the photoconvertible fluorophore mEos3.2 were cultured for three days. Then a small MT patch in the axon was photoconverted at an average 32% neurite length towards the axon tip and the summed photoconverted intensity measured for each timepoint. **(a)** Illustration of photoconversion experiments and differences of axons compared to neurites that were implemented in simulations. **(b)** Representative neuron for **d,e**. Intensity is color-coded from purple (low) to yellow (high). Time is in min:seconds. Scale bars, 20 μm for not zoomed and 4 μm for zoomed images. **(c)** Decay curve of summed photoconverted intensity with fitted dual exponential function of representative neuron in **b**. **(d-e)** Decay curves were fitted to dual exponential function and **(d)** half-life and **(e)** fraction of stable MT mass were plotted for neurites and axons. For neurites and axons in **d**, three and six datapoints with a half-life above 10,000 min are out of the plotting range, respectively. (neurite data from Fig. 1,e-f; **d**: neurite: $n = 38$ neurons, axon: $n = 32$ neurons, $p = 0.00099$; **e**: neurite: $n = 45$ neurons, axon: $n = 32$ neurons, $p = 1.7 \times 10^{-5}$; $N = 4$ experiments for all groups, Mann-Whitney U test). **f,g**, Comparison of experimental data from **d** and **e** and simulations over 160 μm for **(f)** stable MT half-life and **(g)** fraction of stable MT mass obtained from fitted dual exponential curve ($n = 500$ simulations, simulation time: 1660 min). To fit simulation results to experimental data, the stable MT loss rate is lowered (from 0.15 min^{-1} to 0.07 min^{-1}) and stabilization rate is increased (from 0.04 min^{-1} to 0.14 min^{-1}). **h-j**, Simulations with **(i)** slow MT-RF in 3-day-old axons of $0.1 \mu\text{m/min}$ and **(j)** fast MT-RF of developing neurites of $0.5 \mu\text{m/min}$ and with high ($5.0 \mu\text{m}^{-1}$) or low ($2.8 \mu\text{m}^{-1}$, as in simulations of developing neurites and dendrites) resources for MT branching ($n = 500$ simulations, simulation time: 1600 min). **(h)** Average number of distal MTs from simulations in **i,j** from 128 μm (80%) to 144 μm (90%). **(i,j)** Distribution of unstable and stable MTs with the region used to calculate distal MTs marked by dashed lines. **k**, Comparison of the distribution of stable and unstable MTs from experiments and simulations from **i** with a high amount of MT branching resources. Values were normalized to the middle of the axon. For the experimental

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distributions, neurons cultured for three days were stained for tyrosinated and acetylated or tyrosinated and detyrosinated tubulin and the corresponding distributions were measured for 100 μm to 200 μm long axons. Intensities were quantified for each point along the axon by summing up all pixel intensities across the axon width at that point and normalizing the value by the point closest to the soma. The distribution of non-acetylated tubulin was calculated as in Fig. 1I and used as the distribution for unstable MTs, while the distribution of acetylated tubulin was used as the distribution for stable MTs (Acetylated: $n = 56$ neurons; $N = 3$ experiments). The thick line in boxplots indicates the mean, the thin line the median. Due to the large number of simulations, model and experimental data are not statistically compared and thus only statistically significant differences between experimental data are shown (Methods section “Statistics”).

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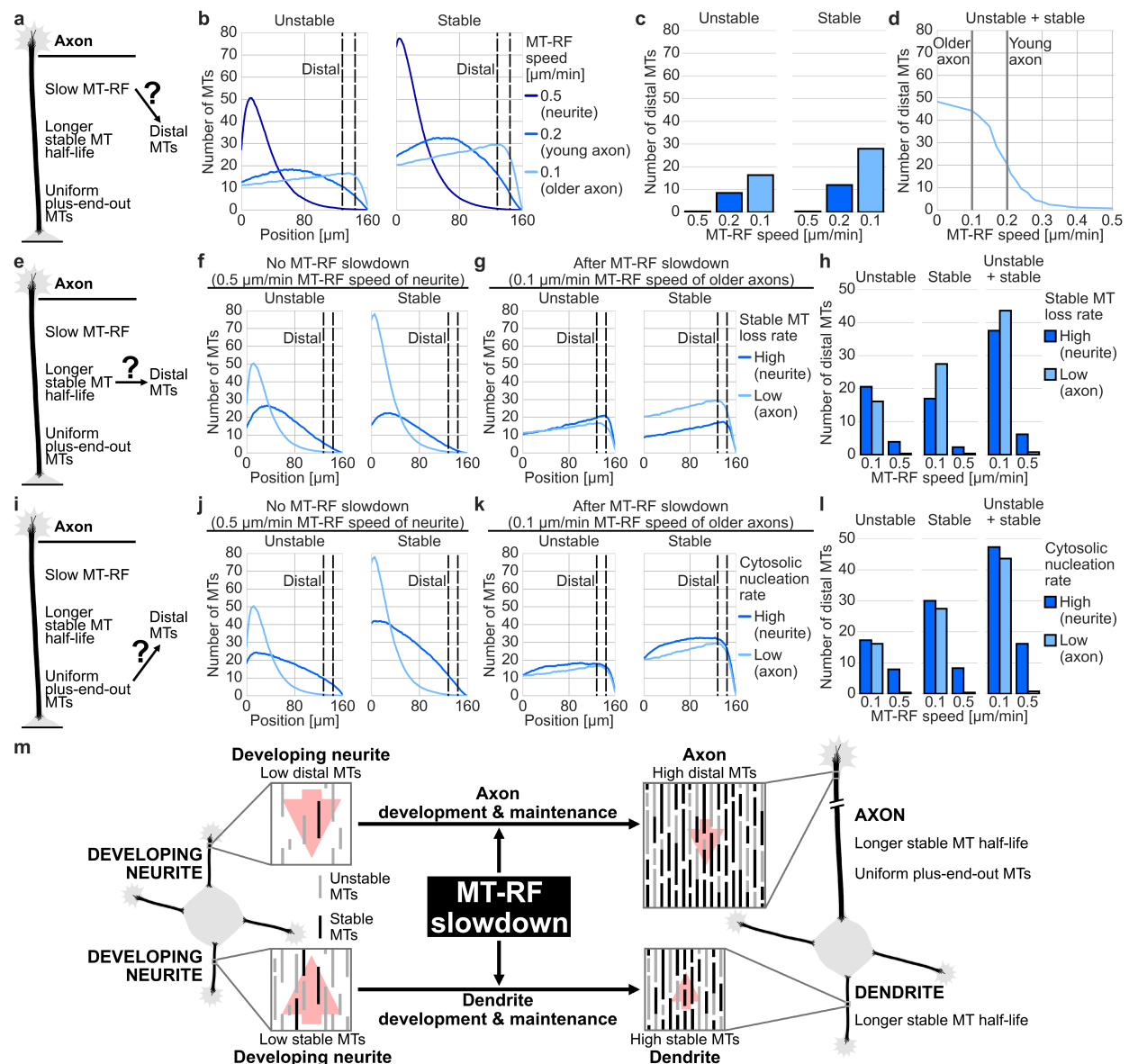


Fig. 7: MT-RF slowdown is needed for distal MTs due to axonal MT properties

a-l, Stable, unstable and the sum of stable and unstable MTs are quantified from simulations over 160 μm (**b,f,g,j,k**) across the entire region. Dashed lines in **b,f,g,j,k** indicate the distal region from 128 μm (80%) to 144 μm (90%) used to calculate distal MTs for the analyses in **c,d,h,i**. (**a-d**) Simulations with MT-RF speed from developing neurites (0.5 μm/min), young axons (0.2 μm/min) or 3-day-old axons (older axons, 0.1 μm/min) (n = 500 simulations, simulation time: 1600 min). (**a**) Changes implemented in the axon based on experimental data. Slow MT-RF could influence distal MTs in the axon. (**b**) Distribution of unstable and stable MTs. (**c**) The average number of distal unstable and stable MTs for different MT-RF speeds. (**d**) The average number of distal MTs depending on the MT-RF speed. MT-RF speed of 1-day-old axons (young axon; 0.2 μm/min) and 3-day-old axons (older axon, 0.1 μm/min) are marked by grey lines. (**e-h**) Simulations with combinations of the fast stable MT loss rate of developing neurites (0.15 min⁻¹) or the slow stable MT loss rate of axons (0.07 min⁻¹) with slow axonal MT-RF (0.1 μm/min) or fast MT-RF (0.5 μm/min) (n = 500 simulations, simulation time: 1600 min). (**e**) Slow stable MT loss rate could influence distal MTs in the axon. (**f,g**) Distribution of unstable and stable MTs for (**f**) fast MT-RF and (**g**) slow MT-RF. (**h**) The average

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1591 number of stable, unstable and total distal MTs for fast and slow stable MT loss rate. (i-l)
 1592 Simulations with the low cytosolic nucleation rate fitted to the fraction of plus-end-in MTs in
 1593 Extended Data Fig. 6a-c ($0.003 \text{ min}^{-1}\mu\text{m}^{-1}$) or the high cytosolic nucleation rate as used in
 1594 developing neurites and dendrites (Figs. 2-5; $0.2 \text{ min}^{-1}\mu\text{m}^{-1}$), combined with slow MT-RF of 0.1
 1595 $\mu\text{m}/\text{min}$ or fast MT-RF of $0.5 \mu\text{m}/\text{min}$ were run ($n = 500$ simulations, simulation time: 1600
 1596 min). (i) The high proportion of plus-end out MTs could influence distal MTs in the axon. (j,k)
 1597 Distribution of stable and unstable MTs for (j) fast MT-RF and (k) slow MT-RF. (l) The average
 1598 number of stable, unstable and total distal MTs. m, MT-RF slowdown increases distal MTs in
 1599 the axon due to the increased effect and stable MTs in dendrites to drive the development of
 1600 both axons and dendrites.

1601 **Table 1: Standard parameter values used for key simulations.**

Parameter	Value for neurites (Figs. 2 to 4)	Value for dendrites (Fig. 5)	Value for axon (Fig. 6)	Source of value	Description
v_g	17 $\mu\text{m}/\text{min}$	17 $\mu\text{m}/\text{min}$	17 $\mu\text{m}/\text{min}$	Literature ^a	MT growth speed
v_s	7 $\mu\text{m}/\text{min}$	7 $\mu\text{m}/\text{min}$	7 $\mu\text{m}/\text{min}$	Experiment ^b	MT shrinkage speed
v_{ge}	9.47 $\mu\text{m}/\text{min}$	9.47 $\mu\text{m}/\text{min}$	10.0 $\mu\text{m}/\text{min}$	Calculated ^c	Net MT growth speed
v_f	0.5 $\mu\text{m}/\text{min}$	<u>0.1 $\mu\text{m}/\text{min}$</u>	<u>0.1 $\mu\text{m}/\text{min}$</u>	Literature/ Experiment ^d	MT-RF speed
k_{pause}	10.8 min^{-1}	10.8 min^{-1}	10.8 min^{-1}	Experiment ^e	Pausing rate (Growing unstable to pausing unstable state)
k_{rescue}	2.78 min^{-1}	2.78 min^{-1}	2.78 min^{-1}	Calculated ^f	Rescue rate (Pausing unstable to growing unstable state)
$k_{\text{cat}}^{\text{base}}$	0.69 min^{-1}	0.69 min^{-1}	0.69 min^{-1}	Fitted ^g	Unstable MT loss rate
$k_{\text{cat}}^{\text{change}}$	0.69 $\text{min}^{-1}\mu\text{m}^{-1}$	0.69 $\text{min}^{-1}\mu\text{m}^{-1}$	0.69 $\text{min}^{-1}\mu\text{m}^{-1}$	Fitted ^h	Linear increase of k_{cu} , from $d_{\text{cat}}^{\text{change}}$ distance to neurite tip per μm
$d_{\text{cat}}^{\text{change}}$	2 μm	2 μm	2 μm	Literature ⁱ	Distance from neurite tip where the linear increase in k_{cu} starts
k_{loss}	0.15 min^{-1}	<u>0.05 min^{-1}</u>	<u>0.07 min^{-1}</u>	Fitted ^j	Stable MT loss rate
k_{stable}	0.04 min^{-1}	0.04 min^{-1}	<u>0.14 min^{-1}</u>	Fitted ^k	Stabilization rate (Pausing unstable state to stable state)
k_{growth}	0.5 min^{-1}	0.5 min^{-1}	0.5 min^{-1}	Fitted ^l	Stable growth rate (Stable state to growing stable state)
k_{cyto}	0.2 $\text{min}^{-1}\mu\text{m}^{-1}$	0.2 $\text{min}^{-1}\mu\text{m}^{-1}$	<u>0.003 $\text{min}^{-1}\mu\text{m}^{-1}$</u>	Fitted ^m	Cytosolic nucleation rate
k_0	5.5 min^{-1}	5.5 min^{-1}	5.5 min^{-1}	Fitted ⁿ	Somatic nucleation rate
k_{branch}	1.7 $\text{min}^{-1}\mu\text{m}^{-1}$	1.7 $\text{min}^{-1}\mu\text{m}^{-1}$	1.7 $\text{min}^{-1}\mu\text{m}^{-1}$	Fitted ^o	MT branching rate per μm MT length
res	2.8 μm^{-1}	2.8 μm^{-1}	<u>5 μm^{-1}</u>	Fitted ^p	Resources for MT branching
$d_{\text{removal}}^{\text{soma}}$	5 μm	5 μm	5 μm	Literature ^q	Distance into the soma after which MTs are removed

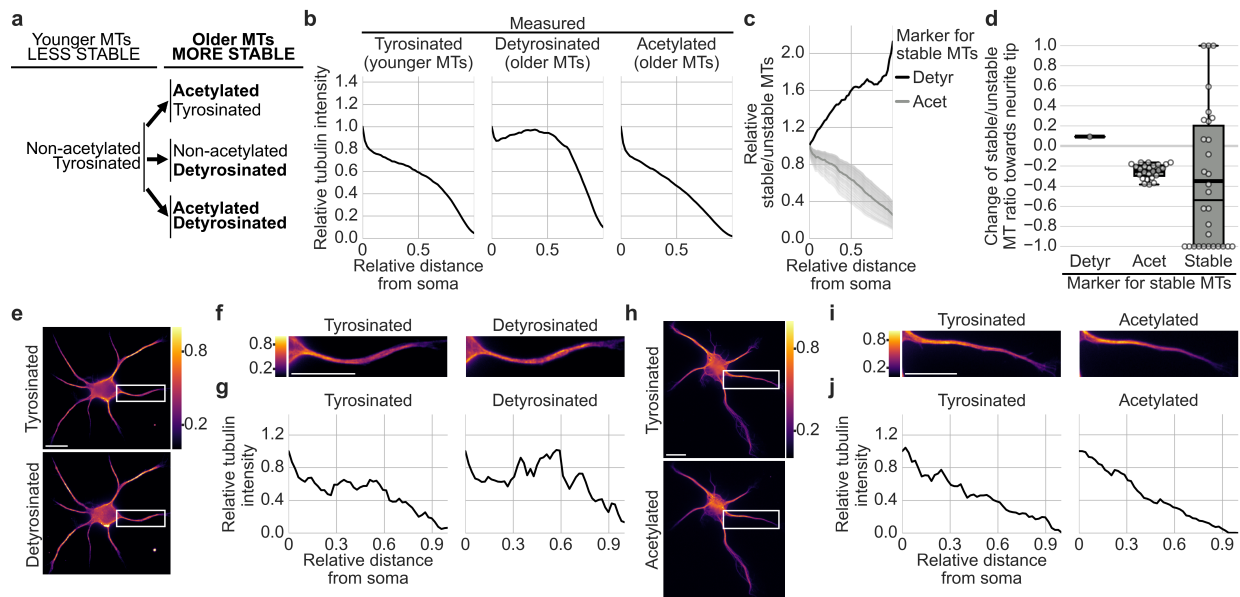
1602 Parameter values for axon and dendrite simulations that were different from the parameter
1603 value set for neurites due to the fit to new experimental data were underlined. **a**, ¹⁹ **b**,
1604 Measured in growth cone (Extended Data Fig. 2f-g), with 3 $\mu\text{m}/\text{min}$ subtracted due to
1605 retrograde MT movement in growth cone³⁴. **c**, Calculated from equation (B13) (Methods). The
1606 slight difference in v_D for axon simulations comes from the change in k_{pspu} , which affects v_D

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1607 through k_{GuPu} calculation in equation (B13) (Methods). **d**, ¹⁶ and Extended Data Fig. 5a-c. **e**,
 1608 Measured in Extended Data Fig. 2a-c. **f**, Calculated from equation (B10). **g**, Fitted to data in
 1609 Fig. 3f. **h**, Fitted to data in Fig. 3g, similar increase as measured in²⁴. **i**, Set based on the size
 1610 of small growth cones. **j**, Fitted to data in Fig. 3f for neurites, Fig. 5c for dendrites and Fig. 6f
 1611 for axons. **k**, Fitted to Fig. 3h for neurites and dendrites and Fig. 6g for axons. **l**, Fitted to
 1612 data in Fig. 3i. **m**, Fitted to data in Fig. 3g-i for neurites and dendrites and Extended Data Fig.
 1613 6c for axons. **n**, Fitted to data in Fig. 3h and Extended Data Fig. 2d-e. **o**, Fitted to data in Fig.
 1614 3g-i. **p**, Fitted to data in Fig. 3g-i for neurites and dendrites and Fig. 6k for axons. **q**, Half the
 1615 normal soma size of hippocampal neurons.

1616

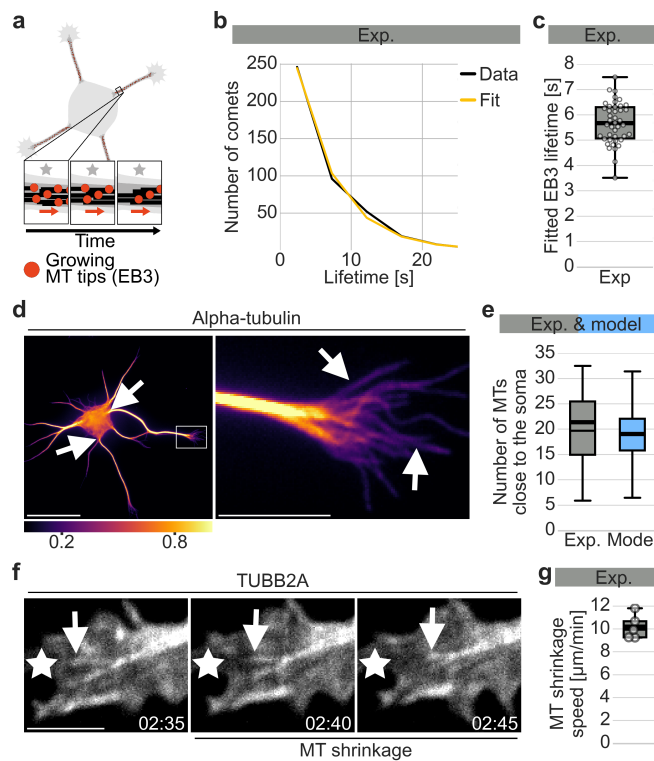
Appendix 2: Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance



Extended Data Fig. 1: Acetylation is an appropriate marker for stable microtubules.

Neurons were stained after one day in culture for tyrosination as a marker for younger and detyrosination as well as acetylation as a marker for older MTs. Intensities were quantified for each point along the neurite as the sum of all pixel intensities across the width of the neurite. Tubulin intensity was normalized to the value closest to the soma. (For Tyrosinated: $n = 1144$ neurites from 331 neurons, $N = 6$; Detyrosinated: $n = 639$ neurites from 188 neurons, $N = 3$; Acetylated: $n = 505$ neurites from 143 neurons, $N = 3$ experiments). **a**, Illustration of MT modifications that neurons were stained for. **b**, Distributions of tubulin modifications were averaged for all neurites. **c**, The distributions of the relative ratio of stable to unstable MTs for detyrosinated tubulin (Detyr) or acetylated tubulin (Acet) as marker for stable MTs. **d**, The normalized change of the ratio of stable to unstable MTs from close to the soma to far from the soma was calculated and normalized by the sum of the ratios at both positions. Detyrosinated and tyrosinated (Detyr) or acetylated and non-acetylated (Acet) tubulin were used as markers for stable and unstable MTs, respectively. This was compared to the value obtained from the data in Fig. 1p as a good marker for stable MTs (Stable). **e-j**, Representative images (**e,h**) of the entire neurons and (**f,i**) the region marked with the white rectangle enlarged, and (**g,j**) the intensity curve along the neurites from **f,i**, stained for (**e,f**) tyrosinated and detyrosinated or (**i,j**) tyrosinated and acetylated tubulin. Scale bars, 10 μm . Intensity in **e,f,h,i** is color-coded from purple (low) to yellow (high). The thick line in boxplots indicates the mean, the thin line the median.

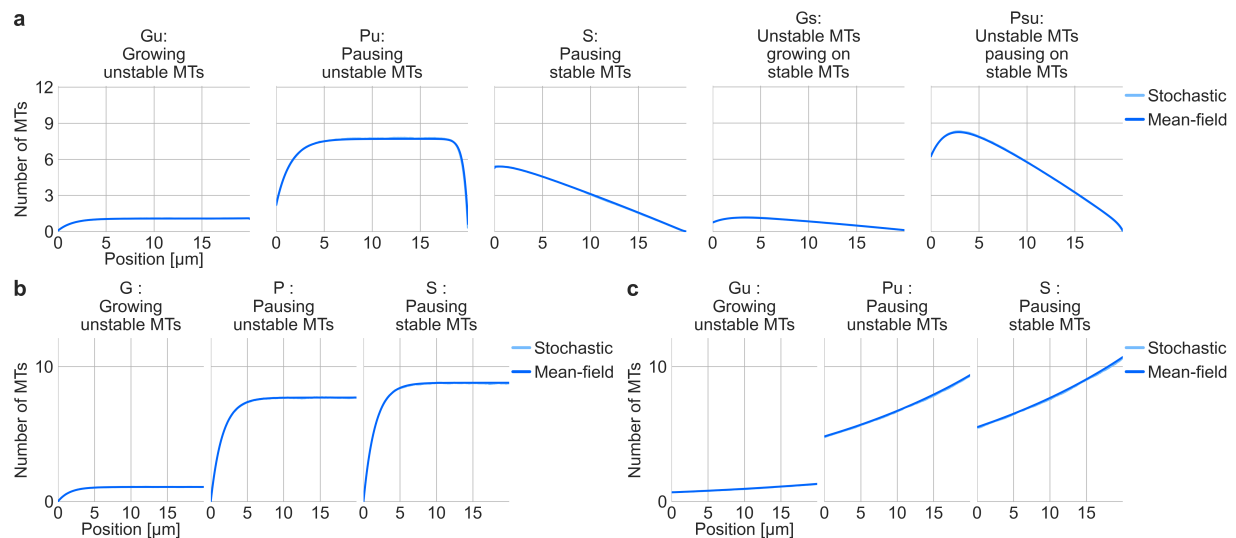
Appendix 2: Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance



Extended Data Fig. 2: Measuring the pausing rate, neurite base MTs and retraction speed.

a-c, Neurons expressing fluorescently labeled EB3 were expressed and imaged after one day in culture in ¹⁶. **(a)** Illustration of imaging growing MT tips. **(b,c)** The histogram of EB3 comet lifetimes was measured from data from ¹⁶. The histogram (Data) was fitted with a mono-exponential function (Fit). **(b)** Histogram of EB3 lifetime for a representative neuron in **c**. **(c)** Fitted EB3 lifetimes (n = 42 neurons, N = 3 experiments). **d,e**, Neurons grown for one day in culture were fixed, stained for alpha-tubulin and imaged. The average intensity of single MTs in growth cones per pixel was measured. The intensity of the neurite at the soma along a one-pixel-wide line was summed and normalized by the average intensity of a single MT per pixel to obtain an estimate of the number of MTs close to the soma. **(d)** Representative neuron with the neurite close to the soma indicated by white arrows on the left side and single MTs in the growth cone indicated by white arrows in the right zoom. Intensity is color-coded from purple (low) to yellow (high). Scale bars, 20 μm for not zoomed and 5 μm for zoomed image. **(e)** Comparison of experimentally measured number of MTs close to the soma and the number of MTs in the model. Simulation data from Fig. 3g (Exp: n = 32 neurons, N = 1 experiment; model: n = 16000 simulations). Due to the large number of simulations, model and experimental data are not statistically compared (Methods section "Statistics"). **f,g**, The growth cone of a neuron expressing the fluorescently labeled tubulin subtype TUBB2a was imaged after one day in culture. **(f)** White arrows indicate a shrinking MT and the star a fixed position. Time is in min:seconds. Scale bar, 2 μm. **(g)** MT shrinkage events were analyzed from the imaged growth cone (n=5 events, N=1 experiment). The thick line in boxplots indicates the mean, the thin line the median.

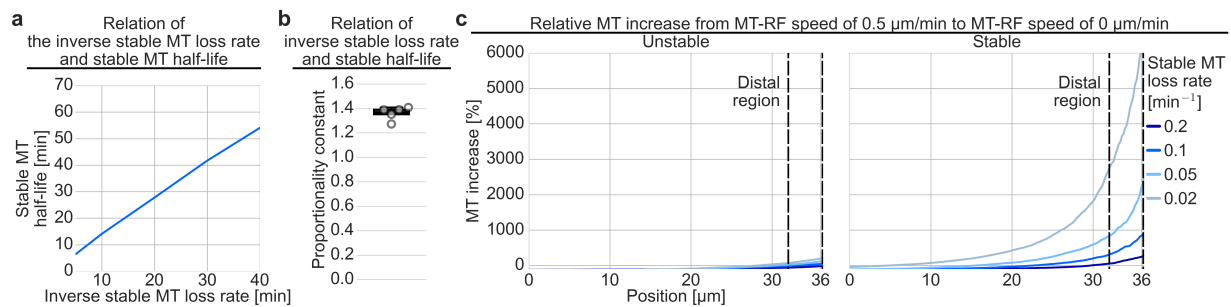
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Extended Data Fig. 3: Stochastic simulations match mean-field solutions.

Mean-field steady-state solutions were calculated from systems of partial differential equations. The equivalent stochastic simulations were run until steady state ($n = 4000$ simulations). **a**, Somatic and MT branching nucleation rates were set to zero. Mean-field solutions were obtained numerically. **b,c**, MT-RF speed was set to zero. Additionally, stable growth rate was set to zero to prevent the formation of unstable MTs growing or pausing on stable MTs. **(b)** Somatic and MT branching nucleation rates. Mean-field solutions were obtained analytically. **(c)** Somatic nucleation rate was set to zero, but MT branching rate was nonzero and not limited by resource availability. Mean-field solutions were obtained numerically.

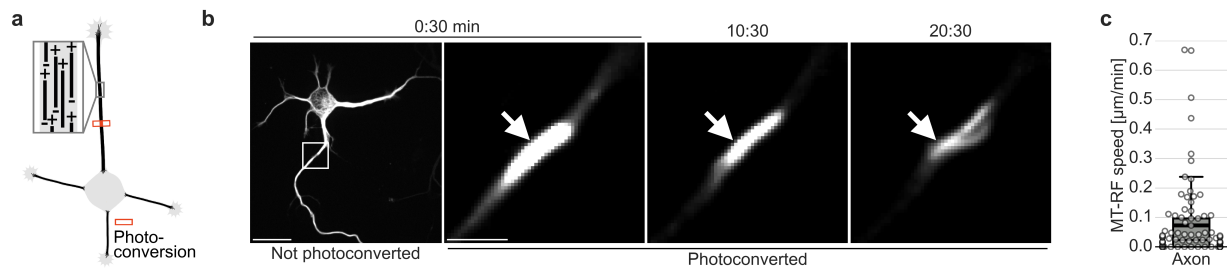
Appendix 2: Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance



Extended Data Fig. 4: Stable MT half-life correlates with inverse stable loss rate and MT-RF slowdown increases MTs the most distally

Simulations over 40 μm with parameters as in Figure 2. **a**, The fitted stable MT half-life is shown for simulations run with different values for the inverse stable MT loss rate. **b**, The proportionality constant of the fitted stable MT half-life divided by the inverse of the stable MT loss rate is shown. ($n = 2000$ simulations, simulation time: 510 min). **c**, The increase in unstable and stable MTs without MT-RF compared to 0.5 $\mu\text{m}/\text{min}$ MT-RF speed is calculated from data in Fig. 2c, d ($n = 2000$ simulations, simulation time: 450 min).

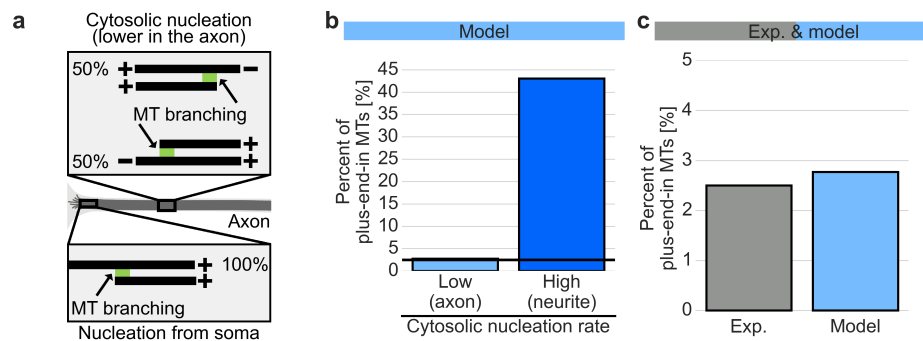
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Extended Data Fig. 5: MT-RF slows further down in old axons.

Neurons expressed the tubulin TUBB2a fused to the photoconvertible fluorophore mEos3.2 and were cultured for three days. Then a small MT patch in the axon was photoconverted on average at 32% neurite length towards the axon tip. **a**, Illustration of photoconversion experiments. **b**, Representative neuron for **c**. The white arrow points to the position of photoconversion. Time is in min:seconds. Scale bars, 20 μ m for not zoomed and 4 μ m for zoomed image. **c**, MT-RF was measured ($n = 46$ neurons, $N = 4$ experiments). The thick line in boxplots indicates the mean, the thin line the median.

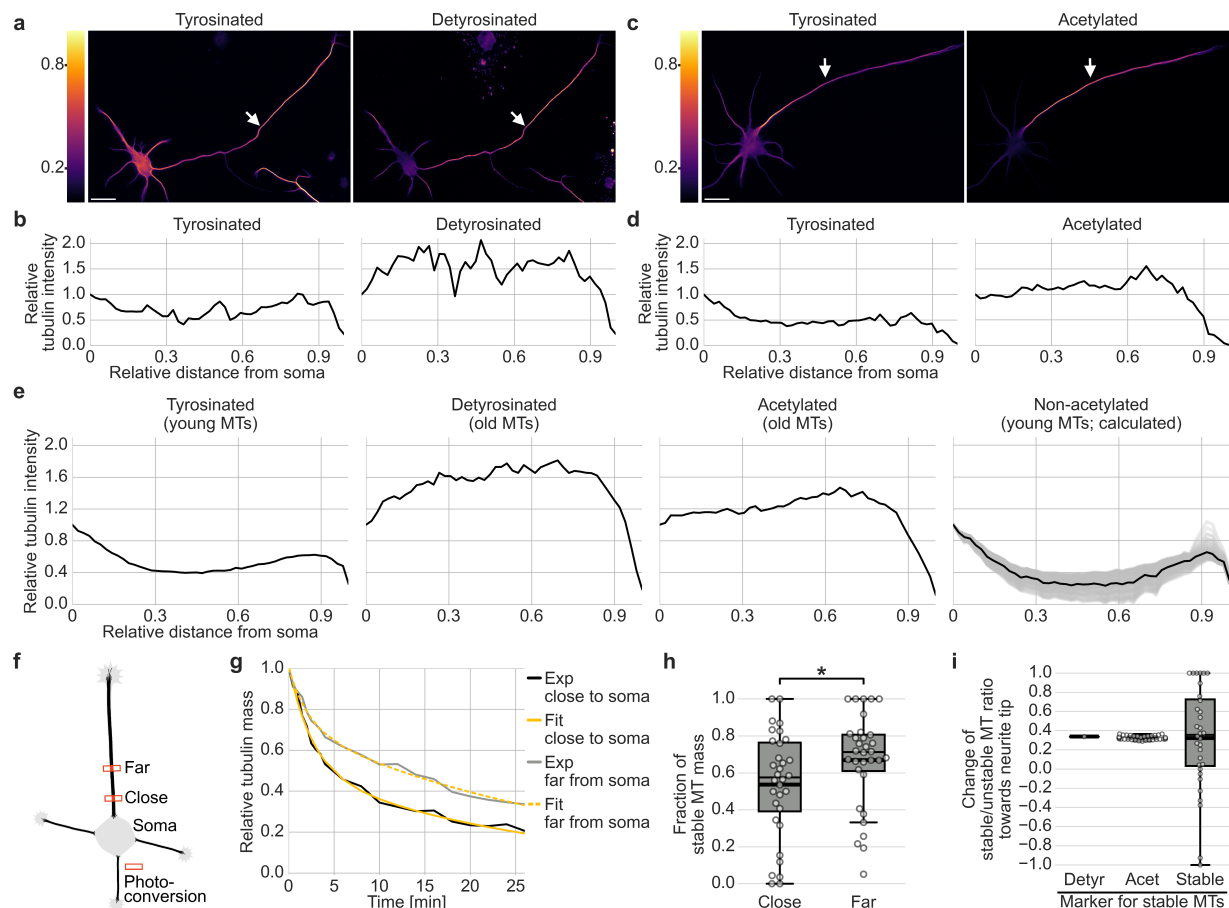
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Extended Data Fig. 6: Percent of model plus-end-in MTs with varying cytosolic nucleation rates.

Simulations with low ($0.003 \text{ min}^{-1}\mu\text{m}^{-1}$) or high ($0.2 \text{ min}^{-1}\mu\text{m}^{-1}$) cytosolic nucleation rate with slow MT-RF of $0.1 \mu\text{m}/\text{min}$ are run ($n = 500$ simulations, simulation time: 1600 min). **a**, Illustration of the influence of nucleation mechanisms on MT orientation and the reduction of cytosolic MT nucleation used in simulations of axons. **b**, The theoretical percentage of plus-end-in MTs is analyzed. The horizontal black line indicates the experimentally expected value of 2.5%. **c**, Theoretical percentage of plus-end-in MTs in simulations with reduced cytosolic nucleation rate (from $0.2 \text{ min}^{-1}\mu\text{m}^{-1}$ to $0.003 \text{ min}^{-1}\mu\text{m}^{-1}$) compared to experimental data of 2.5% plus-end-in MTs from⁵ ($n = 500$ simulations, simulation time: 1600 min).

Appendix 2: Slowdown of microtubule retrograde flow enables axon and dendrite development and maintenance



Extended Data Fig. 7: Distributions of stable and unstable MTs in axons.

a-e, Neurons cultured for three days were stained for **(a,b)** tyrosinated and detyrosinated or **(c,d)** tyrosinated and acetylated tubulin and the corresponding distributions were measured for 100 μm to 200 μm long axons. Intensities were quantified for each point along the axon by summing up all pixel intensities across the axon width at that point and normalizing the value by the point closest to the soma. Intensity in **a,c** is color-coded from purple (low) to yellow (high). **(a-d)** **(a,c)** Images and **(b,d)** intensity curves of representative neurons for **e**. Scale bars, 20 μm . **(e)** Distributions were averaged across axons of all neurons by normalizing the position along the axon. The non-acetylated distributions were calculated from the other distribution as in Fig. 1I (Tyrosinated: $n = 114$ neurons, $N = 4$ experiments; Detyrosinated: $n = 58$ neurons, $N = 4$ experiments; Acetylated: $n = 56$ neurons; $N = 3$ experiments). **f-h**, TUBB2a-mEos3.2 expressing neurons were cultivated for three days, after which an MT patch close to or far from the soma in the axon was photoconverted, on average 12% and 35% axon length towards the axon tip, respectively. For each axon a close and far MT patch were converted separately. **(f)** Illustration of photoconversion experiment. **(g,h)** The photoconverted intensity was measured for each timepoint and a dual exponential function fitted to the decay curve. **(g)** Decay curves for representative neuron. **(h)** Fraction of stable MT mass close to and far from soma ($n = 32$ neurons, $N = 4$; $p = 0.021$, Mann-Whitney U test). **i**, Comparison of the normalized change of the stable/unstable MT ratio from close to the soma to far from the soma for photoconversion experiment (Stable) and when using detyrosinated and tyrosinated tubulin (Detyr) or acetylated and non-acetylated tubulin (Acet) as markers for stable and unstable MTs, respectively. The thick line in boxplots indicates the mean, the thin line the median.

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