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The microtubule cytoskeleton at the synapse

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ABSTRACT

In neurons, microtubules (MTs) provide routes for transport throughout the cell and structural support for dendrites and axons. Both stable and dynamic MTs are necessary for normal neuronal functions. Research in the last two decades has demonstrated that MTs play additional roles in synaptic structure and function in both pre- and postsynaptic elements. Here, we review current knowledge of the functions that MTs perform in excitatory and inhibitory synapses, as well as in the neuromuscular junction and other specialized synapses, and discuss the implications that this knowledge may have in neurological disease

1. Introduction

MTs are polarized cytoskeletal protein filaments, which are comprised of the regulated addition of α- and β-tubulin subunits, preferentially at their fast growing plus end [1,2]. MTs in differentiated neurons are not attached to the centrosome [3], which can lead to a variety of geometric arrays depending on the location of the nucleating material [4,5], as well as the actions of MT severing enzymes [6,7], and molecular motors [8]. In mature axons, MTs are all oriented in the same direction, with the plus ends directed away from the cell body. In dendrites of mammalian neurons, MTs form a mixed polarity of parallel and antiparallel arrangements [9,10].

Neurons possess two pools of MTs, stable and dynamic, with axonal and dendritic MTs having a stable region and a dynamic region, often coexisting on the same polymer [11–13]. Whereas dynamic MTs undergo stochastic transitions from depolymerization to polymerization and vice versa [14], stable MTs remain relatively constant in their polymerized form, resisting depolymerization. Stable MTs represent the majority of the neuronal MT mass. This stability is important for the durable wiring of the nervous system and provides long-lasting support to extensive neuronal structures. During development or in response to signaling, dynamic MTs can be stabilized by MT end capping proteins or by the side binding of MT dependent motors and MT associated proteins (MAPs). Once stabilized, MTs have sufficient longevity to be substrates for tubulin modifying enzymes that, with the exception of acetylated α-tubulin, add molecular moieties preferentially on the carboxyl terminal tails of either the α- or β-tubulin subunit on residues exposed to the surface of the MT lattice. The combinatorial nature of these modifications leads to what has been referred to as the "tubulin code" [15,16], a set of rules, still in the process of being fully understood, which controls a variety of neuronal functions, such as MT remodeling by severing enzymes, kinesin dependent transport, dynein loading at MT plus ends, organelle contacts and further MAP binding [15–29]. Surprisingly, with the exception of tubulin polyglutamylation, whose activity-dependent increase results in slower trafficking of the synaptic protein gephyrin [30], or the presence of a marginal band of modified MTs at retinal bipolar neuron terminals [31], very little is known about the regulation of tubulin PTMs at synapses.

In neurons, MTs are particularly important because they support complex, branching structures, like the dendritic tree and axonal arbor, while maintaining segregation of functional compartments. In addition to providing structural support, MTs act as intracellular highways, creating a roadmap for protein motors to deliver important cargoes to various regions of the cell [3]. While it has long been known that MTs

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support dendritic and axonal structure, their roles at synapses have been explored only over the past decade. Historically, attention to the function of the cytoskeleton at the synapse has been focused on actin. By the 1980s, electron microscopy (EM) studies had demonstrated the presence of MTs in dendritic spines and axonal boutons [32,33], but their involvement at the synapse received new attention only in the late 2000s, when three independent groups reported that MTs enter dendritic spines [34–36]. On the postsynaptic side, dynamic MTs transiently entered dendritic spines in an activity-dependent manner, where they contribute to spine enlargement. With the exception of the neuromuscular junction (NMJ), in which MT disruption had been shown to cause loss of presynaptic organization [37], the role of MTs at the presynaptic side in mammalian neurons has remained uncharted territory until very recently. Current reports indicate that in highly active synapses that require accurate, graded neurotransmitter release, such as ribbon synapses in bipolar neurons of the retina and the Calyx of Held, presynaptic MTs play important roles in synaptic vesicle (SV) cycling and mitochondrial anchoring [31,38,39]. In *en passant* boutons of pyramidal neurons, presynaptic dynamic MTs are nucleated upon neuronal activity and are critical for adjusting activity-evoked neurotransmitter release by providing paths for interbouton bidirectional transport of SVs, which is a rate limiting step in SV unloading and exocytosis at sites of release [40–42].

In this Review, we summarize our knowledge of the emerging, diverse roles that MTs play at pre- and postsynaptic elements in healthy neurons, and the impact that synaptic MT malfunction may have in neurodevelopmental and neurodegenerative disease.

2. The chemical synapse

Since the late 1950s, the ultrastructural features of individual synapses have been studied extensively using snap-shots obtained via electron microscopy (EM). E.G. Gray classified synapses within the brain based on the ultrastructural characteristics of the presynaptic (SV-bearing) and postsynaptic partners (length of apposed membrane, membrane thickenings and synaptic cleft) [32,43–45]. Within the presynaptic axonal bouton, clusters of SVs are prominent, especially near the active zone (AZ), the site of SV docking and neurotransmitter release. Another characteristic feature of the synapse is an accumulation of opaque material on the cytoplasmic face of the postsynaptic membrane, referred to as the postsynaptic density (PSD). The density represents the aggregation of neurotransmitter receptors and signaling proteins essential for chemical synaptic transmission [46].

The presynaptic bouton is an area within the axon specialized for neurotransmitter release [47]. Boutons can be *en passant*, presynaptic regions along the length of the axon, or terminal, at the end of the axon. In response to an action potential, neurons secrete a variety of neurotransmitter molecules from SVs into the extracellular space by exocytosis. Excitatory presynaptic boutons in the central nervous system (CNS) primarily release the neurotransmitter glutamate. The glutamate metabolite γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter released from CNS presynaptic terminals of interneurons [48–50]. Glycine is another inhibitory neurotransmitter that is frequently used in inhibitory synapses, particularly in the spinal cord [51,52].

SV secretion, an event triggered by Ca^{2+} ions, is achieved by the fusion of vesicles with the plasma membrane. Increases in Ca^{2+} levels due to depolarization in the axon cause the SVs to merge with the AZ [47]. This same neuron then retrieves and reassembles the components of the SV, ready to be filled again with the chemical messengers. Organelles like mitochondria and smooth ER can also be present in the bouton, where they can impact synaptic function by regulating energy supply and Ca^{2+} buffering [53–57].

Dendritic spines, tiny protrusions emanating from the dendritic shaft, serve to compartmentalize biochemical and electrical signals and represent postsynaptic sites of excitatory synapses [58–60]. The structure of the dendritic spine is typically a spherical head that contains the PSD and synaptic neurotransmitter receptors, as well as a neck that connects the head to the dendritic shaft. In spine heads, the protein network of the PSD aligns with the site of neurotransmitter release from the presynaptic terminal [61–64]. The PSD serves to cluster glutamate receptors and cell adhesion molecules (CAMs), to recruit signaling proteins, and to anchor these components to the cytoskeleton of the spine [65].

In CNS excitatory synapses, the neurotransmitter glutamate is released from the presynaptic site, and its binding to AMPA receptors (AMPARs), a class of ionotropic receptors, drives an initial, rapid depolarization of the postsynaptic membrane through the influx of Na^{+} and K^{+} ions [66,67]. Glutamate also induces the opening of ionotropic NMDA receptors (NMDARs), but membrane depolarization is necessary to remove Mg^{2+} from blocking the ion channel. Once these conditions are met, depolarizing Na^{+} and Ca^{2+} influx through the NMDARs occurs. Intracellular Ca^{2+} can bind to calmodulin and activate a range of enzymes, such as CaM-KII 67].

Depolarization has long been known to regulate actin dynamics within the spine, leading to enlargement or shrinkage of the spine head [61,67,68]. Indeed, long-term potentiation (LTP) is linked to an increase in spine volume and PSD enlargement [69], while long-term depression (LTD) can result in spine shrinkage [70] and pruning [71]. In general, spines with larger heads have larger PSDs [59], with more AMPARs and NMDARs [72,73]. These findings indicate that larger spine head volumes are linked to greater synaptic strength [74] and that the morphology of spines present on a dendrite can impact neuronal activity and function [75].

3. Postsynaptic MTs at excitatory synapses

It was long believed that dendritic spines contained no MTs, and that actin was the main regulator of spine morphology associated with synaptic plasticity. Although E.G. Grey had published EM images showing MTs residing in both the dendritic spine and presynaptic bouton in 1975 and the 1980's [32,33,44,76], this evidence was overlooked for decades. One explanation for this omission is that Gray used an albumin pre-treatment before fixation that may have allowed the MTs to survive the fixation process, and since this was not a widely used technique, the literature ignored the association of MTs with synaptic contacts. However, recent visualization techniques using EB3-EGFP, a MT binding protein which tracks with polymerizing MT plus ends, unequivocally demonstrated that dynamic MTs can invade dendritic spines (Fig. 1A) at low frequency in cultured neurons [34–36] and organotypic slice cultures [77]. Dynamic MTs penetrate into dendritic spines of different shapes, including mushroom, stubby and thin, as well as filopodia, and silencing of EB3 reduces the frequency of spine invasion [34]. Importantly, drug treatments affecting MT dynamics strongly decreased the total number of spines and could decrease the formation of spines induced by BDNF [34,36].

Several reports support the notion that MT invasion into spines is driven by synaptic activity. NMDAR-dependent synaptic activation in culture and at individual synapses increased the proportion of dendritic spines containing dynamic MTs, which then contributed to spine enlargement [35,36,77,78]. On the other hand, inhibition of NMDAR activity reduced MT invasion of spines [79]. It is thus not surprising that MT invasion is Ca^{2+} dependent, and that Ca^{2+} chelation was shown to reduce MT spine invasions [77,78]. Additionally, dendritic spines exhibiting elevations in Ca^{2+} signaling contain increased amounts of F-actin, and these spines are preferentially targeted by dynamic MTs [77,78]. These observations are compatible with EM images showing new MTs protruding from the dendrite into the spines after tetanic stimulation during LTP [80] and the finding that induction of chemical LTP with high KCl resulted in an increase of dendritic spines that contained MTs at the time of fixation. Moreover, this condition was completely abolished by inhibiting the firing of action potentials with

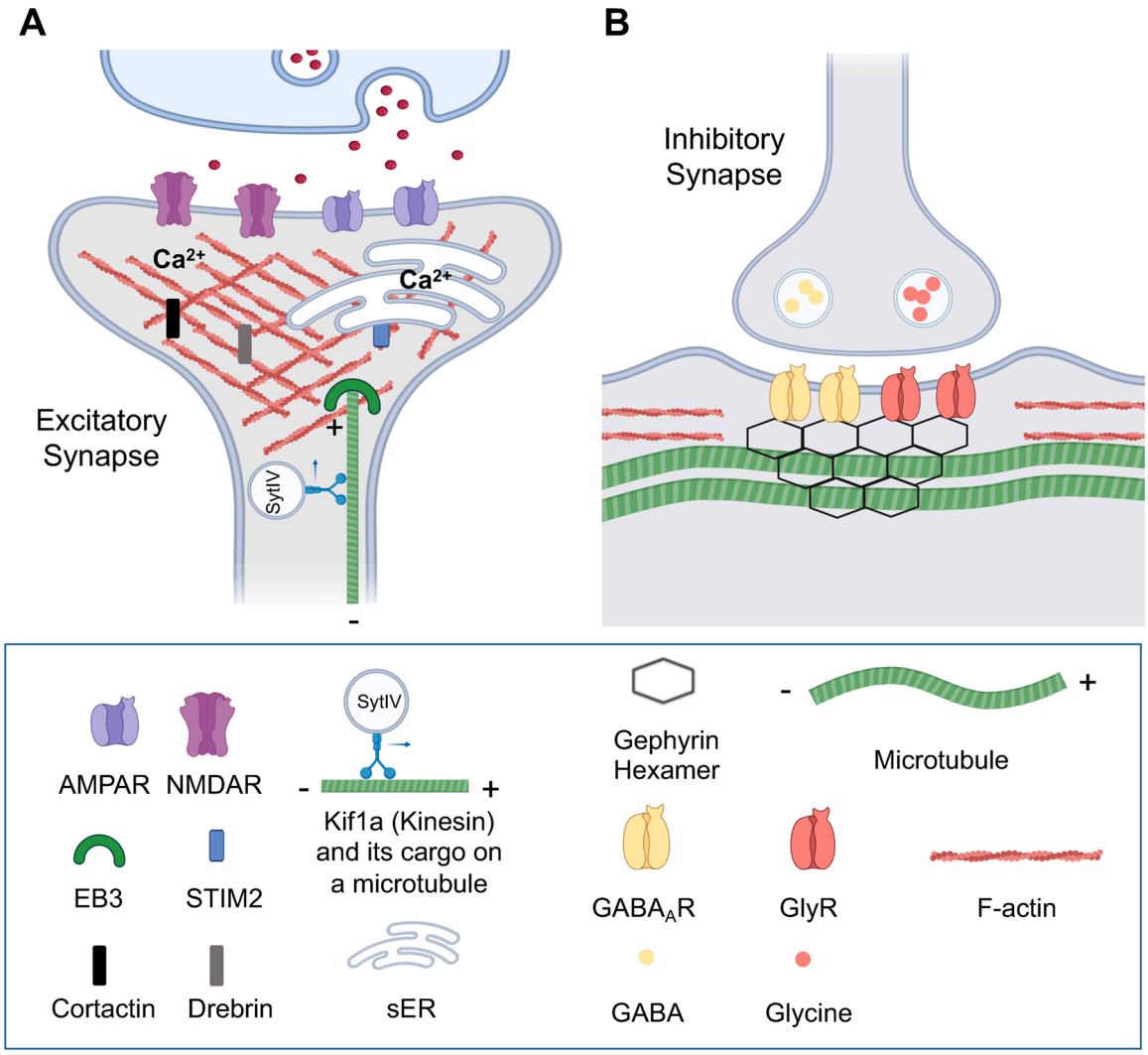

Fig. 1. Schematic of MT functions in two different types of postsynaptic elements. (A) Excitatory postsynaptic site: depolarization of the dendritic spine allows for transient entry of dynamic MTs into the spine. Entry of dynamic MTs into spines has been associated with structural plasticity of the invaded spines. Selective dendritic spine delivery of SytIV is mediated by the MT plus end motor Kif1A. Entry of dynamic MTs into the spine is regulated by the MT plus end binding protein EB3, which can bind to F-actin and F-actin regulators residing in the spine, such as drebrin and cortactin. EB3 is also a binding partner of STIM2, an ER membrane protein and a regulator of Ca^{2+} dynamics in mushroom spines. This binding may provide an additional pathway for entry of STIM2/smooth endoplasmic reticulum (sER) into the spine. (B) Inhibitory postsynaptic site: the postsynaptic element of inhibitory synapses is typically located directly on the dendrite, cell body or axon hillock. Inhibitory synapses can be glycinergic, GABAergic or mixed. Gephyrin acts as a scaffold protein, anchoring glycine and GABA receptors to the microtubule cytoskeleton. While the lateral diffusion of glycine receptors (GlyRs) in the synapse is affected by F-actin, lateral diffusion outside of the synapse is controlled by MTs, a mechanism that may be important for the dynamic regulation of the neuronal membrane "apparent viscosity" to control the "influx" and "efflux" of receptors at the synapse during synaptic plasticity.

the voltage-gated Na^{+} channel blocker tetrodotoxin (TTX) [35].

It is not fully understood how MTs target spines from the dendritic shaft, but a role for F-actin has been proposed. MT plus end binding proteins ($^{+}$TIPs), such as EB3, have been shown to interact with F-actin, and activity-evoked F-actin at the base of the spine may provide a pathway through which recently active spines can be targeted [77]. On the other hand, MT penetration into spines may influence their morphology by affecting the actin cytoskeleton. Indeed, whereas the spine head increases after MT invasion, a reduction of EB3 impairs spine invasion and synapse development [34–36,77].

It was initially proposed that EB3-positive MT ends influenced spine morphology by altering the turnover of p140Cap, an adaptor protein that acts as a hub for many postsynaptic proteins. In particular, p140cap could affect the actin cytoskeleton through the regulation of Src kinase activity and its substrate cortactin, an actin stabilizer and nucleation promoting factor [36]. A later study further implicated drebrin, a developmentally regulated actin binding protein that promotes the formation of stable F-actin. Drebrin can also bind EB3, which allows it to act as an actin/MT cytoskeleton coordinator [81], and was originally reported to be necessary and sufficient to promote MT invasions of dendritic spines [78]. However, this observation was challenged by a subsequent study indicating that loss of drebrin expression did not affect MT invasion of spines, but rather that cortactin and the ARP2/3 complex were the key players required for dynamic MT entry into spines [77]. Regardless of this controversy, it is clear that regulation of F-actin is important for this process, as drebrin, cortactin and the ARP 2/3 complex all promote actin polymerization.

It is conceivable that dynamic MTs invading into spines serve as preferential tracks for synaptic cargo delivery in addition to diffusion and myosin-dependent transport. The invasion of dynamic MTs into dendritic spines may allow MT-dependent motors to deliver specific cargoes that are essential for synaptic plasticity, including the PSD core protein, PSD-95 [82]. Dynamic MTs may also regulate synaptic plasticity by delivering recycling endosomes containing AMPARs into spines

from the dendritic shaft [83]. However, given that under basal conditions the frequency of MT-spine invasions is relatively low, it is likely that AMPAR transport into spines is mostly mediated by actin and that MT dependent spine entry is only restricted to a few spines through an unknown mechanism of selection.

Except for synaptotagmin IV (syt-IV) (Fig. 1A), a postsynaptic protein that regulates synaptic function and LTP, very little is known about the nature of both dendritic spine MT motors and their cargos. Indeed, McVicker et al. (2016) showed that the kinesin Kif1A can deliver syt-IV to dendritic spines via transient MT invasions [84]. However, silencing of Kif1A resulted in more syt-IV exocytosis at extra-synaptic sites on dendrites, suggesting that delivery of this cargo through dynamic MT entry may be necessary to restrict transport of syt-IV to activated synapses. The study also reported that Kif1A was not required for mitochondrial entry into spines, implying that mitochondrial delivery occurs only through actin/myosin handoff [84], a mechanism also implicated in delivery of the endoplasmic reticulum (ER) to synapses [85]. In the case of the ER, however, it is possible that growing MT ends may contribute to Ca^{2+}-regulated ER entry into synapses by allowing the tracking of the Ca^{2+} sensors and ER resident stromal interaction molecules 1 and 2 (STIM1/2) through EB1/3 binding [86,87] (Fig. 1A).

Altogether, this emerging evidence convincingly demonstrates that dynamic MTs are recruited to spines upon neuronal activity to deliver specific cargos and that $^{+}$TIPs interactions with F-actin allow specific spines to be targeted by pathways that impact spine morphology and synaptic plasticity [36,88,89].

4. Postsynaptic MTs at inhibitory synapses

Unlike many excitatory synapses, most inhibitory synapses are not sequestered at a spine, but instead form synapses directly on a dendrite, soma or axon initial segment. γ-GABA and glycine (Gly) are the two common inhibitory neurotransmitters used in the CNS and are typically released by a class of cells called interneurons. One of the functions of interneurons is to control the firing of glutamatergic pyramidal cells. By precisely directing pyramidal cell activity, interneurons can regulate network activity, generate oscillations, and even terminate pathological hyperexcitability [90,91].

Unlike excitatory synapses in dendritic spines, the postsynaptic elements of inhibitory synapses are directly anchored to the MT cytoskeleton via adaptor proteins. Gephyrin is the major scaffolding protein that organizes the postsynaptic density of inhibitory synapses by anchoring Gly receptors (GlyRs) and $GABA_A$ receptors ($GABA_ARs$) to the MT cytoskeleton and neurofilaments through its binding to polymerized tubulin [92], the β subunit of GlyRs [93], and the 1, 2 and 3 α subunits of $GABA_ARs$ [94]. For $GABA_ARs$, the presence of the γ2 subunit is also important for gephyrin-related postsynaptic clustering [95]. Gephyrin not only has a structural function at synaptic sites, but also plays a crucial role in synaptic dynamics and is a platform for multiple protein-protein interactions, bringing receptors, cytoskeletal proteins and downstream signaling proteins into close spatial proximity [94,96,97].

Since gephyrin acts as a scaffold to cluster $GABA_ARs$ [95,98], it is thus not surprising that the clustering of $GABA_ARs$ at synapses has also been shown to depend on the presence of an intact MT cytoskeleton (Fig. 1B). Acute application of nocodazole to depolymerize MTs did not directly interfere with $GABA_AR$ function [99,100] but considerably altered the organization of $GABA_AR$ clusters at the plasma membrane [95,101].

The roles for the MT cytoskeleton in GlyR function remains controversial, although a few studies support the idea that MT alteration can induce changes in the organization of the postsynaptic gephyrin scaffold and alter GlyR stabilization at synapses [102–104]. Gephyrin binds with high affinity and cooperativity to tubulin and MTs [92] and *in vitro* depolymerization of MTs by the alkaloid demecolcine reduced the percentage of cells with postsynaptic gephyrin clusters and the number of clusters per cell in spinal neurons [102]. Additionally, demecolcine treatment dispersed synaptic GlyR clusters so that only a few GlyR clusters co-localized with presynaptic vesicle markers, suggesting that MTs may directly regulate the lateral mobility of the gephyrin/GlyR complex in the postsynaptic membrane [102]. In contrast, however, MT disruption in hippocampal cultures maintained in culture for up to 28 days failed to affect gephyrin/$GABA_A$ clusters, casting doubt on the contribution of tubulin in gephyrin positive inhibitory synapses [105]. Interestingly, van Zundert et al. (2004) found that the severity of the de-clustering in response to the MT depolymerization was dependent on the age of the neuronal culture, and was more severe in immature cultures (DIV 7) than more mature cultures (DIV 10–12). By DIV 17, gephyrin and GlyR clustering were no longer affected by depolymerization of the MT cytoskeleton. This suggests that the inhibitory effect of alkaloid-mediated MT disruption on immature glycinergic synapses may be reliant on expression of the neonatal α2β GlyR but not the adult α1β GlyR 104]. In agreement with this hypothesis, glycinergic miniature postsynaptic currents (mIPSCs), which occur in response to spontaneous release of glycine via SV fusion from a presynaptic site, also became insensitive to colchicine with the maturation state of spinal neurons [106]. It is however conceivable that the more dynamic MT cytoskeleton found in developing neurons plays an important role in anchoring clusters during neuronal differentiation that is absent in later developmental stages, or that the more stable and modified MT cytoskeleton in older neurons might be more resistant to depolymerization. Further work is necessary to address these questions.

Synaptic plasticity at inhibitory synapses relies on the lateral diffusion of neurotransmitter receptors at synapses, which depends on the interaction of synaptic receptors with submembrane scaffolding proteins. Interestingly, both the actin and MT cytoskeletons were shown to play a role in the plasticity of inhibitory synapses through their impact on receptor lateral diffusion, a process regulated by the state of postsynaptic differentiation and the properties of the extrasynaptic membrane [107]. Using single particle tracking, Charrier et al. found that disruption of either the MT or actin cytoskeleton increased GlyR exchange between synaptic and extrasynaptic membrane regions and decreased the time that the receptor spent at the synapse [107]. Interestingly, while the lateral diffusion of GlyRs was affected by actin inside the synapse, lateral diffusion outside of the synapse was controlled by MTs, compatible with a model by which direct contact with the MT cytoskeleton is critical for the dynamic regulation of the neuronal membrane “apparent viscosity” to control the “influx” and “efflux” of receptors at inhibitory synapses during synaptic plasticity [107].

5. Presynaptic MTs at excitatory synapses

The presence of tubulin in subcellular fractions from nerve endings and its association with the presynaptic membrane was first reported in the early 1970s [108–110]. In addition, tubulin directly interacts with the presynaptic proteins synapsin I, synaptotagmin I and α-synuclein [111–114], suggesting a functional association between MTs, SV clustering and neurotransmitter release. E.G. Gray was the first one to show that MTs were present in the presynaptic axonal boutons from mammalian cerebral and cerebellar cortices [44], and recent findings continue to support Gray’s observations. The postsynaptic dendritic spines in apposition to presynaptic boutons indicate that Gray was examining excitatory synapses in fixed forebrain tissue [32]. In the bouton, a population of MTs appeared to anchor to the AZ membrane. These MTs, which were typically covered in SVs organized in clusters, were found to attach to the membrane within the AZ, indicating that they may serve as an SV organizer in these synapses, as well as a source of direct tracks to attachment sites at the AZ [32]. Gray also described a set of MTs that formed a marginal coil in the bouton, which was closely associated with mitochondria [32] (Fig. 2A). These early observations suggested that in the CNS MTs can serve as tracks for intra and inter-bouton SV delivery and also as structures for organizing mitochondrial

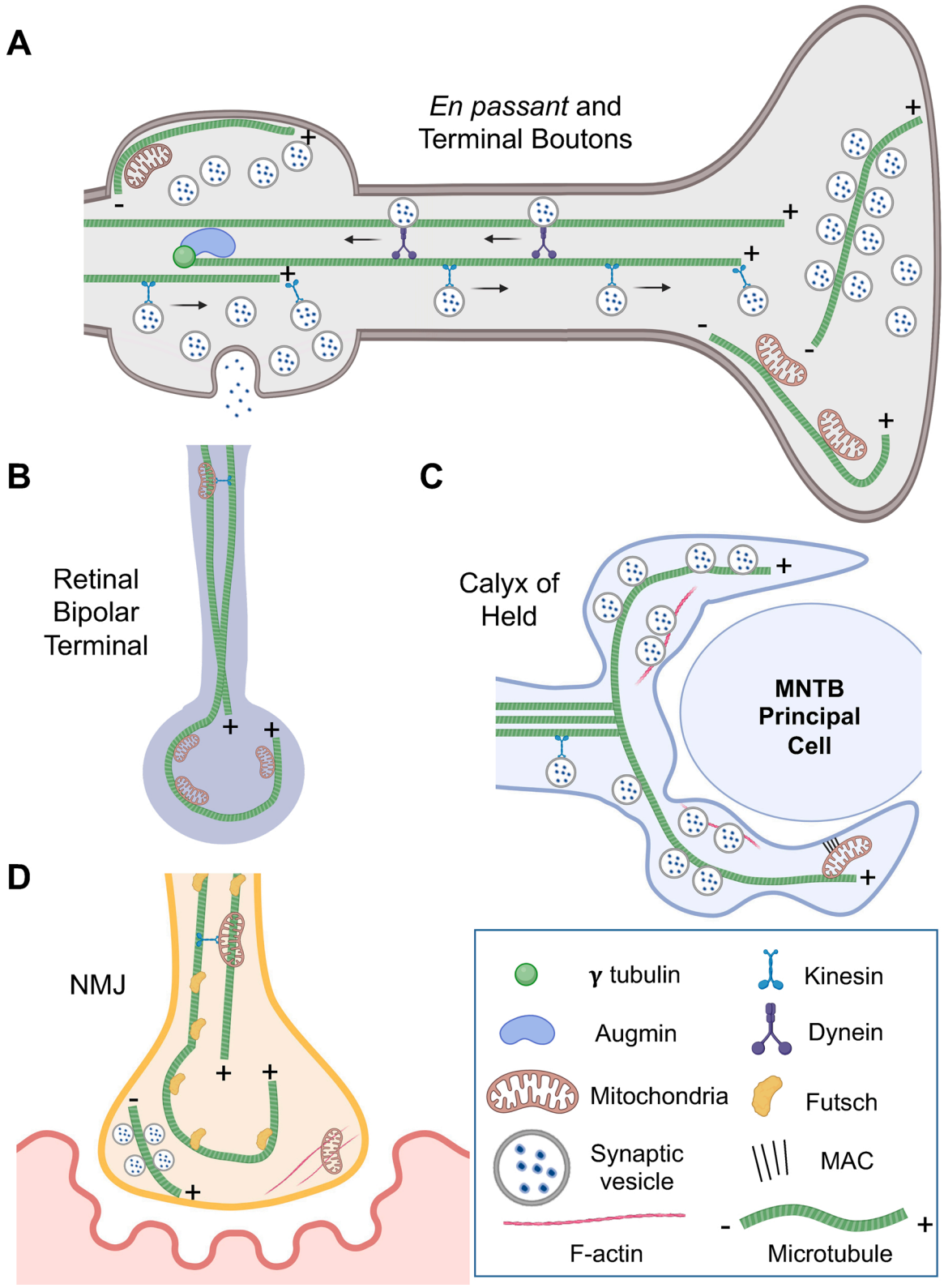

Fig. 2. Schematic of MT functions in different types of presynaptic elements. (A) Excitatory presynaptic bouton: by EM, MTs can be found associated with mitochondria and SVs close to the active zone. Functional studies have ascribed a role for γ-tubulin and augmin *de novo* nucleated MTs at presynaptic *en passant* boutons in the regulation of neuronal transmission by limiting the rate of bidirectional interbouton SV transport and Kif1A-mediated SV delivery and unloading to sites of release. (B) In the goldfish retinal bipolar neurons, MTs loop into the presynaptic bouton to organize and anchor mitochondria in the presynaptic area. (C) In the mammalian auditory CNS, the giant calyx of Held synapse surrounds the soma of the MNTB (Medial Nucleus of the Trapezoid Body) principal cell. In calyceal terminals, presynaptic MTs extend throughout the presynaptic area and organize SVs and MAC superstructures. MTs also play essential roles in inter-synaptic movements of SVs that are rate limiting for high-frequency neurotransmission. (D) In the presynaptic bouton of the NMJ, MTs form a loop in the presynaptic area, which is stabilized by Futsch/MAP1B. This loop is important in the budding process of newly forming boutons. Synaptic vesicles have also been observed on MTs that approach the active zone.

placement at the synapse. In agreement with these findings, both synaptosomes and intact axon terminals from cerebral cortex were found to contain horseshoe-shaped mitochondria encircled by three to ten MTs opposite the synaptic membrane [115].

In addition, emerging evidence supports a critical role for MTs in mitochondrial organization in goldfish retinal bipolar neurons and the mammalian giant calyceal terminals of Held (Fig. 2B and C). Retinal bipolar cells are specialized glutamatergic CNS neurons, that like other neurons of the visual and auditory systems, need to communicate graded, prolonged signals that enable accurate and rapid SV exocytosis [116]. Bipolar cells are constantly active and adjust their tonic release behavior according to inputs received from photoreceptor cells. In these neurons, a structure known as the synaptic ribbon is thought to help fulfill this purpose. The synaptic ribbon is a protein scaffold that holds a readily releasable pool of SVs, nanometers away from voltage gated calcium channels. A single ribbon can organize large numbers of SVs, and this large pool allows for high rates of continuous release [117–119]. MTs were found to approach the synaptic ribbon, but did not appear to organize SVs [43]. However, MTs seemed to play a role in mitochondrial organization by forming a marginal band that encircles the periphery of the presynaptic terminal, separate from the synaptic ribbon. Mitochondria were described to be highly associated with this loop of stable and modified MTs and inhibition of kinesin activity prevented mitochondria from accumulating at the terminal. These findings suggest that in these highly active neurons MTs are necessary for maintaining appropriate numbers of mitochondria at the bouton in order to supply the high energy required for presynaptic function [31].

Extensive MT structures have also been observed in the largest synapse of the mammalian brain, the Calyx of Held. The Calyx of Held is a specialized presynaptic glutamatergic terminal that, like the retinal bipolar synapse, must relay sustained and graded signals via SV exocytosis [120,121]. Electron tomography and tubulin immunolabeling in presynaptic preparations from Calyx of Held synapses indicated that MTs contribute to the anchoring of mitochondria to the presynaptic membrane via the mitochondrion associated adherens complex (MAC) superstructure, suggesting that also in these large synapses the MT cytoskeleton participates in localizing mitochondria at sites of high metabolic demand [122]. Using confocal and high-resolution microscopy, Babu et al. recently described that MTs inserted fully into calyceal terminal swellings and partially colocalized with a subset of SVs [38]. Short term depression (STD) is a form of synaptic plasticity that occurs after prolonged activity depletes readily releasable SVs [123]. Recovery from STD is temporally divided into two phases: fast and slow. While F-actin depolymerization delayed the fast-recovery component of EPSCs from short-term depression, depolymerization of MTs prolonged the slow-recovery time. The exact mechanisms behind the slow recovery component are not well understood, but one explanation involves the movement of vesicles from the reserve pool to the readily releasable pool [38]. The reserve pool can supply SVs to the readily releasable pool and also helps to prevent soluble bouton proteins from diffusing into the axon [124–127]. Additionally, automatic tracking of large populations of fluorescently labeled vesicles within calyceal presynaptic terminals in culture has shown that MTs play essential roles in inter-synaptic movements of SVs that could be rate limiting for high-frequency neurotransmission [39].

In agreement with these findings, a couple of recent manuscripts support the notion that presynaptic MTs may provide the tracks for inter-bouton SV transport also in smaller presynaptic terminals of pyramidal neurons [40,41]. Using live-cell microscopy and single-molecule reconstitution assays, Guedes-Dias et al., demonstrated that in cultured hippocampal neurons, the localized enrichment of dynamic MTs at *en passant* boutons specifies an unloading zone to ensure the accurate delivery of SV precursors by the kinesin-3 KIF1A motor to control presynaptic strength 41]. In agreement with this, Qu et al., reported that in primary hippocampal neurons, excitatory *en passant* boutons are hotspots for the nucleation of dynamic MTs on demand [40]. Presynaptic *de novo* MT nucleation depended on γ-tubulin and the augmin complex, which was required for correct MT polarity. Importantly, MT nucleation occurred at excitatory boutons in hippocampal slices from neonatal mice, was induced by neuronal activity, and controlled glutamate release by providing dynamic tracks for targeted interbouton transport of SVs (Fig. 2A).

Altogether, these results show that in mammalian CNS synapses presynaptic MTs: 1) contribute to maintaining the capability for high frequency neurotransmitter release, 2) can be controlled by neuronal activity and 3) play an important role in the regulation of both intra and interbouton SV trafficking.

6. Presynaptic MTs at the neuromuscular junction

Much of the contemporary understanding of synaptic form and function was derived from studies of the neuromuscular junction (NMJ) [128,129], and the NMJ is commonly used to study synaptic MTs in the peripheral nervous system [130,131]. The NMJ synapse is comprised of a motor neuron whose axon synapses with muscles cells, forming a branched synaptic terminal arbor with a large number of synaptic boutons. The typical neurotransmitter at NMJ synapses is acetylcholine, but there are many studies of the glutamatergic larval *Drosophila* NMJ [132].

At the *Drosophila* NMJ presynaptic terminal, MTs form thread-like loops that extend into the bouton [37,133] (Fig. 2D). In addition to that, a subset of dynamic MTs regulated by the formin *Diaphanous* and known as “pioneer presynaptic MTs”, protrudes into the presynaptic terminal and controls synaptic growth [134]. During the development of presynaptic boutons, presynaptic MT loops go through a dynamic restructuring that requires MTs splaying apart into numerous fibers and then re-bundling after the new bouton begins to bud [37,133]. Like other types of synapses, MTs at the vertebrate NMJ have been found to both organize SVs and approach the active zone [135]. However, mitochondria have not yet been observed in close association with the MT loop, casting doubt on the conserved nature of this functional feature among different types of synapses. Indeed, while mitochondria are found at the presynaptic site of NMJs, it appears that the actin cytoskeleton may play a more dominant role in mitochondrial organization, at least in vertebrates [136].

While other studies have shown a role for MTs in the AZ, the NMJ has been very useful for identification of some of the MT binding partners and regulators. Futsch, a MAP1B homolog in *Drosophila*, is a MT binding protein that promotes MT stability of the MT loops at presynaptic boutons [37,133,137,138]. Importantly, Futsch acts as a linker between presynaptic MTs and components of the AZ [139], and presynaptic MT dynamics are regulated by post-translational modifications of Futsch. For instance, phosphorylation of Futsch by Shaggy (Sgg) causes Futsch to lose affinity for MTs and detach, leading to destabilization of the presynaptic MT cytoskeleton [140,141]. Conversely, calcineurin, a protein phosphatase that acts on phosphorylated Futsch at normal Ca^{2+} levels, counteracts Sgg, and promotes MT stability [142]. Genetic interaction studies consistently link the formin DAAM with the Wg/Ank2/Futsch pathway of MT regulation and bouton formation [143–146]. A recent study reported that DAAM is tightly associated with the synaptic AZ scaffold, and electrophysiological data point to a role for DAAM in the modulation of SV release [147]. Based on these results, the authors propose that DAAM is an important cytoskeletal effector of the Wg/Ank2 pathway involved in bouton formation and synaptic MT organization by coupling the AZ scaffold to the presynaptic MT cytoskeleton.

Altogether, these findings demonstrate that in the NMJ, MTs not only contribute to the development of the presynaptic element but also to presynaptic function, paving the way for further exploration of the roles of NMJ presynaptic MT organization in SV release.

7. Synaptic MTs in neurodevelopmental and neurodegenerative disease

The morphological plasticity of dendritic spines is inextricably linked to learning and memory [148], and spine abnormalities characterize AD, schizophrenia and developmental neurological disorders such as Fragile X and Down Syndrome [148–151]. Interestingly, indirect measurements of MT stability in synaptosomal fractions of mice subjected to single-shock contextual fear conditioning have associated MT stability/instability phases with learning and memory formation, indicating that regulation of synaptic MTs may play a primary role in plasticity, aging and dementia related disorders [152,153]. Consistently, inhibition of MT dynamics was recently reported in neurons from kif21b KO mice that exhibit learning and memory disabilities [154].

Loss of MT integrity and spine density are major pathological features of AD [155–158]. However, recent studies have suggested that hyperstabilization of dynamic MTs, rather than global MT destabilization, may initiate AD pathology and related disorders. In hippocampal neurons, for instance, oligomeric Aβ promoted acute stabilization of dynamic MTs and this activity was mediated by mDia1, a formin regulating both presynaptic activity and MT stabilization, and was associated with tau-dependent spine loss [159]. In AD, tau becomes hyperphosphorylated and binding to the MT cytoskeleton is highly reduced. A recent study supports the notion that tau allows for a longer labile domain on MTs [160] and loss of tau expression would promote MT binding of MAP6, an intraluminal MAP that protects MTs from cold-induced depolymerization by inducing neuronal MTs to coil [161–164]. Since dynamic MTs are necessary for dendritic spine invasions and interbouton SV transport after neuronal activity, reduction in the labile domain of MTs could have severe effects on the ability of dynamic MTs to invade synapses with negative consequences on neurotransmission and synaptic plasticity. Interestingly, MAP6 also directly regulates spine morphology by interacting with actin [165] and loss of MAP6 in mice recapitulates cognitive defects observed in schizophrenia [166–168], suggesting that a balance between MAP6 and tau may be critical to maintain proper cytoskeletal dynamics in neurons and that this balance is necessary to avoid synaptic disease.

Dysfunctional MT dynamics at synapses may also affect plasticity by altering Ca^{2+} buffering through regulation of mitochondrial and ER anchoring. Indeed, EB binding to STIM proteins are implicated in the regulation of Ca^{2+} channels as part of the store-operated ER calcium entry (SOCE) pathway induced by intracellular Ca^{2+}-store depletion [169,170]. STIM1 is also involved in the regulation of nerve terminal Ca^{2+} influx by affecting voltage-gated Ca^{2+} channel activity [171,172]. Interestingly, STIM2 levels are lower in AD, and while STIM2 overexpression protects mushroom spines from amyloid beta peptide toxicity *in vitro* and in *vivo*, EB3 overexpression rescues loss of mushroom spines resulting from STIM2 depletion [173,174]. The role of MT dynamics at spines in this functional compensation is unknown.

The fly NMJ has been an ideal model system for the study of human diseases related to neurodegeneration and neuromuscular dysfunctions [175]. About 40 % of all autosomal dominant cases of hereditary spastic paraplegia (HSP) map to the gene that encodes human spastin, a MT severing enzyme related to katanin [176–178]. In *Drosophila*, spastin is enriched in presynaptic terminals at NMJs where it controls MT stability, modulating synaptic structure and function [179]. The role of mutant spastin in the regulation of presynaptic MTs in mammalian neurons is unknown.

The fragile X mental retardation protein (FMRP) is an RNA-binding protein encoded by the *FMR1* gene that represses transcription of selected mRNAs. The absence of FMRP results in fragile X syndrome, which is one of the leading causes of inherited mental retardation [180]. In fragile X, there is abnormal dendritic spine maturation, both in patients [181] and in Fmr1 KO mice [182]. In addition to mRNAs encoding for proteins regulating spine morphology [180], FMRP represses the translation of Futsch/MAP1B, and this repression is necessary for proper synaptic development [183–185]. High levels of MAP1B during this critical period result in increased MT stability and improper synaptogenesis 185], providing another example of how MT hyperstabilization may lead to synaptic disease. Interestingly, Futsch is also a substrate of the leucine-rich repeat kinase 2 (LRRK2), a protein implicated in familial forms of Parkinson's disease (PD). Futsch mRNA binds to the trans-active response DNA binding protein (TDP-43), a nuclear protein that forms aggregates in amyothophic lateral sclerosis (ALS) [186,187]. It is unknown whether this regulation also occurs in mammalian neurons.

8. Conclusions and future directions

MTs play a variety of roles in the development and maintenance of synapses. While in a general sense they support all neuronal functions because they provide a trafficking highway inside the cell, they have specific roles at presynaptic and postsynaptic sites. In dendritic spines, dynamic, transient invasions are induced by neuronal activity and necessary for spine maintenance and plasticity. On the postsynaptic side of inhibitory synapses, MTs serve as an important anchor and contribute to synaptic plasticity. Presynaptically, they impact interbouton SV dynamics and through their relationship with mitochondria residing at boutons, may ensure appropriate ATP levels and Ca^{2+} buffering for proper neurotransmitter release. Despite this compelling evidence, many questions remain to be addressed. Since MT invasion of spines is linked to activity, identification of additional cargos to recently depolarized spines via dynamic MTs remains an attractive area of investigation. For example, it is still unclear whether lysosomes or MAP2 and MAP6 [188,189], all recruited to synapses upon activity, also utilize dynamic MT tracks to get into or away from the synapse. In addition, local protein synthesis has been observed to occur in spines upon activity [190]. RNA granules, which contain mRNA, and large and small subunits of ribosomes are normally transported by Kif5 along dendritic shafts. Whether RNA granules, ribosomal subunits or polysomes are trafficked into spines via activity-evoked MT invasions remains to be determined. It is also unclear whether MT entry into spines represents a default pathway for terminating dendritic MT growth [77] or if it further depends on local on demand MT nucleation from MTs residing in the dendritic shaft. At presynaptic sites, on the other hand, in addition to a potential role for more stable MTs in anchoring presynaptic organelles, we still need to decipher the rules that regulate *de novo* dynamic MT nucleation at selected *en passant* boutons and whether these MT pathways are conserved in other types of CNS synapses and/or required for the interbouton delivery of specific clusters of SVs or rate-limiting presynaptic components.

In large synapses, future work will be needed to determine the specific role that MTs play in organizing mitochondria and perhaps other organelles at presynaptic terminals and whether this spatial regulation affects individual ribbon synapses and graded synaptic transmission.

Given their emerging role in synaptic function, it will soon become critical to determine whether defective MT structure and dynamics at the synapse cause spine atrophy and bouton degeneration observed in both neurodevelopmental and neurodegenerative disease and whether restoring the synaptic MT cytoskeleton may be sufficient to prevent or normalize circuit dysfunctions.

Author contributions

Julie Parato and Francesca Bartolini wrote and edited the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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