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Microtubules in Dendritic Spine Development and Plasticity

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Abstract: Recent studies indicate that microtubules (MTs) may play an important role in spine development and dynamics. Several imaging studies have now documented the exploration of dendritic spines by dynamic MTs in an activity-dependent manner. Furthermore, it was found that alterations of MT dynamics by pharmacological and molecular approaches exert profound influence on the development and plasticity of spines associated with neuronal activity. It is reasonable to speculate that dynamic MTs may be responsible for targeted delivery of specific cargos to a selected number of spines and/or for interacting with the actin cytoskeleton to generate the structural changes of spines associated with synaptic modifications.

Keywords: Synapse, postsynaptic terminals, synaptic plasticity, cytoskeleton, cytoskeletal dynamics, actin.

DENDRITIC SPINES AND SYNAPTIC PLASTICITY

Dendritic spines are tiny membrane protrusions that serve as the postsynaptic terminals for most of the excitatory synapses in the vertebrate brain. The spine head contains the postsynaptic density (PSD) that features clustered neurotransmitter receptors, scaffolding components, and signaling proteins, all of which are positioned on the immediate opposite side of presynaptic terminals for effective reception of neurotransmitters. There is an overwhelming amount of evidence to show that synapses are plastic and undergo short- and long-term modifications during developmental refinement of neural circuits, as well as in learning and memory [1-3]. Furthermore, many neurological disorders have been associated with alterations of synaptic connections [3, 4]. Therefore, a better understanding of the molecular and cellular mechanisms underlying synaptic plasticity is of importance to our understanding of brain development and functions under both physiological and pathological conditions.

Modulation of synaptic strength involves both pre- and post-synaptic terminals, of which the presynaptic changes are often associated with an adjustment in the probability of neurotransmitter release [5]. On the postsynaptic side, modifications of the number and activity of surface neurotransmitter receptors are considered to be a key event underlying synaptic modification. For example, the intracellular domain of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) is phosphorylated to increase ion conductance during early long term potentiation (LTP) [6]. To generate a long-lasting LTP, however, it is necessary to increase the number of postsynaptic glutamate receptors on the postsynaptic surface. These postsynaptic changes appear to be reversed during long term depression (LTD), including dephosphorylation of AMPARs and their removal from the postsynaptic membrane. While changes in transmitter recepiors directly contribute to the modification of synaptic strength, many studies have reported that morphological changes of the postsynaptic terminals are equally important for postsynaptic modulation of synaptic strength. There is a large amount of literature documenting the enlargement of spine heads as well as the emergence of new spines after the induction of LTP, whereas spine shrinkage and elimination are considered to be a key step in LTD [7-9], which have been postulated to be the structural basis of memory [10-12]. Importantly, these morphological/structural changes in spines have been observed during development, aging, learning and neurodegenerative diseases [10-12].

THE CYTOSKELETAL ARCHITECTURE OF DENDRITIC SPINES

A hallmark of dendritic spines is their enrichment in actin, which is believed to be the major player for spine morphology and dynamics. Live-cell imaging studies in vitro and in vivo have established that spines are plastic and undergo activity-dependent changes in morphology, which are believed to be controlled by the actin cytoskeleton. Actin polymerization is coupled with spine enlargement/formation during LTP, whereas LTD involves spine shrinkage through actin depolymerization [8, 9, 13]. Furthermore, many actin-associated regulatory proteins, as well as signaling pathways targeting the actin cytoskeleton, have been implicated in spine dynamics and development [14-16].

Unlike actin filaments, microtubules (MTs) are not believed to play a direct role in spine dynamics, primarily due to the fact that they, although concentrated in dendritic shafts, are rarely seen in spines by standard light and electron microscopy. MTs are polarized hollow tubules from a head-to-tail assembly of the α/β-tubulin heterodimers. The plus end of MT favors polymerization whereas the minus end favors disassembly; the latter however is often capped by centrosomal or other minus end capping proteins. As a result, MTs generally grow from their plus ends in cells but they often switch from growth to rapid shrinkage and vice versa. It is believed that this feature of MT plus ends, termed dy-

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dynamic instability, represents an important mechanism by which MTs could reassemble into different structures under-
varying cell functions. For example, the dynamic insta-

cility could enable MTs to explore various cellular locations
for potential interacting structures and signaling components;
a productive interaction with specific cellular struc-
tures/signals could result in stabilization of the MTs and fur-
ther recruitment of MTs and other cellular structures. In
cells, the structure and dynamics of MTs are regulated by a
large number of MT-associated proteins (MAPs) that often
bind along the entire length of MTs. Significantly, recent
studies have discovered several classes of proteins that spe-
cifically bind to the plus ends of MTs to regulate their sta-

bility, as well as to enable coupling to other cellular structures
[17]. In neurons, MTs in both axonal and dendritic processes
are present as parallel bundles crosslinked by MAPs, which
not only provide the structural foundation for the polarized
neuronal morphology but also serve as the tracks for MT-

motor-based long-range transport of cellular cargos. MTs in
axons have unidirectional arrangement with the plus end
pointing to the distal end of the axon, whereas MTs in den-
drites exist in mix polarity. It is known that these MT para-

meters with the plus end

lent at the lead-
ing edge but shorten at the cell body and the rear [19]. In
fibroblasts, the growth of MTs at the leading edge was found
to activate Rac1 GTPase, which in turn induces actin poly-

merization for lamellipodial protrusion [20]. In nerve growth
cones, early immunostaining studies suggested that MTs do
not enter the actin-rich peripheral region of the growth cone
consisting of filopodia and lamellipodia, leading to a similar
view that the actin cytoskeleton is primarily responsible for
growth cone steering [21]. However, live imaging of fluo-

rescently tagged tubulin proteins in growth cones showed that
MTs enter and explore the peripheral region of the growth cone
[22]. Further studies using sophisticated high-resolution
live microscopy have convincingly shown that MTs enter filo-
opodia and actively interact with actin filaments in growth
cone filopodia to influence growth cone motility [18, 23].
Furthermore, direct local modification of MT dynamics has
been shown to be sufficient to induce growth cone turning
through the Rho GTPases and actin cytoskeleton [24]. While
migrating cells and growth cones may be different from
spines in respect to their cytoskeletal dynamics and regula-
tion, it is tempting to speculate that the actin-based spine
dynamics may be influenced by MTs.

MTs IN DENDRITIC SPINES: EARLY EVIDENCE

The notion that MTs might be present in postsynaptic
terminals first came from electron microscopic studies of
synapses in brain tissues. With the aid of enhanced MT pres-
servation techniques, Westrum and Gary showed that MTs
appear to associate with the postsynaptic density [25]. Their
subsequent EM study further revealed that some MTs are
present between disks of smooth endoplasmic reticulum
(ER), a special organelle referred to as spine apparatus, in
large spines of some pyramidal neurons [26]. MTs were also
found in the proximal apical dendritic spines of hippocampal
CA3 pyramidal neurons, which are usually large and branched,
and contain subcellular organelles including ribo-

somes, multivesicular bodies (MVB), and mitochondria [27].
In the CA1 region of hippocampal slices, MTs were also
detected in dendritic spines, but it was thought to be a possi-
ble artifact from recovery after slice preparation [28]. The
possible existence of MTs in spines was also supported by
immuno-EM detection of β-tubulin and the MT-associated
protein 2 (MAP2) in postsynaptic density, although whether
these tubulin proteins polymerize into MT structures was
unknown [29].

Besides EM studies, a number of biochemical studies
have also implicated the presence of MTs in dendritic spines.
For example, proteomic studies using two-dimensional gel
electrophoresis of tryptic peptides in conjunction with differ-
ent identification methods found tubulin in postsynaptic den-
sity fractions from whole brain [30-33]. In addition, tubulin
mRNA was found in synaptosomal preparations enriched for
dendritic spines [34]. The physical interaction between solu-
ble forms of tubulin, such as tubulin heterodimers, and post-
synaptic proteins, such as NMDAR subunits, has also been
presented [35]. Other indirect evidence came from the detec-
tion of MAP2 in the postsynaptic region, and that activation
of NMDAR decreased its phosphorylation [36]. However,
evidence for the lack of MAP2 in spines was also reported
[37, 38]. It is possible that different fixation conditions em-
ployed in these studies might have contributed to these con-

cflicting results. In addition to MAP2, several other MT-

binding proteins were also implicated to participate in post-
synaptic events. Light chain 2 of MAP1 is a strong interactor
in the postsynaptic stargazin-AMPA receptor complex, pos-
sibly involved in AMPA receptor trafficking prior to inser-
tion at synapse [39]. Another protein, CRIP7 (cysteine-rich
interactor of PDZ three), was identified as a PSD component
and binds specifically to PSD95. With its ability to bind tu-
bulin, CRIP7 may regulate PSD95 interaction with MTs
[40]. Finally, the observation of several types of membra-

uous organelles (e.g. mitochondria) in spines also suggests a
possible presence of MTs in spines since many of these or-
ganelles are primarily transported by MT-dependent motors.
It should be noted, however, that many membraneous organ-
elles can also move along actin filaments via myosin motors.
It is thus plausible that MT-based long-range transport of
these organelles could be switched to actin/myosin-based
trafficking for spine delivery of these organelles. Further
studies are clearly required to elucidate the precise traffick-
ing mechanisms for many of these organelles and vesicles.

MTs IN DENDRITIC SPINES: NEW EVIDENCE

A recent EM study found that strong tetanic stimulation
used for LTP induction caused a significant increase in MTs
entering dendritic spines of hippocampal CA1 neurons [41].
These MTs were seen to bear a characteristic hollow tubular
shape with a diameter of 25 nm, indicating that they are in-
deed polymerized MTs. The marked increase of MTs in spines after tetanic stimulation also suggests a potential function for MTs in activity-dependent modification of postsynaptic structures and functions. However, the limitation of EM made it difficult to quantify the extent of dendritic spines that were visited by MTs, as well as the temporal nature of these MTs. It was also not clear if the observed redistribution of MTs into spines had any significant effects on the spine dynamics and synaptic functions. These important questions have now been partially addressed by three recent studies that employed high-resolution fluorescence live microscopy in conjunction with pharmacological and molecular manipulations of MT dynamics [42-44].

The first major conclusion from the three studies is that dendritic spines do contain MTs, but the percentage of spines containing MTs at a given time point is quite low, likely due to their very dynamic nature. In our study, we used confocal imaging to examine the presence of MTs in spines of cultured hippocampal neurons expressing both GFP-tubulin and mOrange [42]. The z-sectioning and 3D reconstruction by confocal imaging allowed us to more accurately capture MTs in spines at different focal planes of a specific segment of the dendrite. To assure that we are detecting polymerized MTs, we excluded the GFP-tubulin signals in thin spines because we were unable to determine if they represent soluble GFP-tubulin proteins that filled up the thin spines. With this stringent selection criterion, we consistently detected MTs in a small but significant number of spines: about 4% of mushroom spines. The low percentage of spines containing MTs is consistent with the other two studies involving live imaging of GFP-tagged tubulin or EB3, a member of MT Plus-end Tracking Proteins (+TIPs) associated with the plus ends of growing MTs [45]. While only a small number of spines were seen to contain MTs at a given time point, long term time lapse imaging using total internal reflectance fluorescence microscopy (TIRFM) showed that a much larger portion, if not all of dendritic spines, were explored by dynamic MTs over time [43]. Importantly, these MTs only transiently stayed in spines with an average time of a few minutes. It is this transient nature of dynamic MTs in spines that likely contributed to the small percentage of spines captured containing MTs at a fixed time. Interestingly, it was found that the invasion of MTs into spines was stimulated by neuronal activity. Repetitive membrane depolarization using KCl increased the number of dendritic protrusions containing MTs, as well as the average time of MTs staying in spines. These results are consistent with the EM study and suggest that MT distribution in dendritic spines is regulated by neuronal activity.

What is the consequence of dynamic MTs entering dendritic spine? This question was partially answered by the time-lapse live imaging in which the entry of fluorescently-tagged MTs into spines was found to be followed by growth and enlargement of the particular spines [43, 44]. Further support for MTs on spine shape came from pharmacological and molecular manipulation of MT dynamics. Inhibition of MT polymerization or EB3 knockdown reduced the number of mushroom-shaped spines, while increasing the number of filopodia-like dendritic protrusions, without affecting the total number of dendritic protrusions. On the other hand, overexpression EB3-GFP in neurons resulted in an opposite effect: an increase in mushroom-shaped spines together with a reduction in dendritic filopodia-like protrusions. Since dendritic spines are thought to develop and mature from filopodia-like protrusions to mushroom-like spines during synaptogenesis [46], these data suggest that MTs play an important role in spine development and maturation. Indeed, our data showed that shRNA knockdown of EB3 reduced the number of mature spines in hippocampal neurons, suggesting the importance of MT dynamics in spine formation.

The development of spines and synapses is regulated by a variety of extracellular factors and neuronal activity [10]. Brain-derived neurotrophic factor (BDNF) is one of the neurotrophins known to promote spine development and synapse formation [47]. To test a role of MTs in spine development, we used MT-specific drugs (nocodazole for disrupting and taxol for stabilizing dynamic MTs, respectively) to manipulate MT dynamics and examined the spine development of a particular neuron under the influence of BDNF over several days by live confocal imaging. To avoid a gross disruption of MTs in the cells, both nocodazole and taxol were used at very low concentrations (in nanomolar range), which have been shown to primarily affect the dynamic ends of MTs without causing disruption of the overall integrity of existing MT networks [24]. The observation that the baseline level of spine formation in hippocampal cultures was not affected by these MT-drugs at low concentrations support the notion that MTs were not substantially disrupted. Interestingly, we found that the low dose of taxol further potentiated the effects of BDNF on spine formation, leading to about 30% more spines than those treated with BDNF alone. On the other hand, nocodazole completely blocked the effect of BDNF. Taxol with BDNF also significantly increased spine size, implying an acceleration of spine maturation by MT stabilization during BDNF exposure. These findings suggest that MTs play an important role in spine development under neurotrophic influence.

The mechanism by which MTs increase spine size and formation is believed to be mediated by the actin cytoskeleton [44]. It was shown that EB3 regulation of spine morphology was correlated with changes in actin polymerization within the spines. Furthermore, knockdown of EB3 caused a loss of F-actin from dendritic protrusions and conversely, overexpression of EB3 increased F-actin abundance. Moreover, treatment of neurons with jasplakinolide, a drug that binds and stabilizes actin filaments, rescued the EB3 knockdown phenotype to increase mushroom-shaped spines. On the other hand, treatment of neurons with latrunculin B, an actin monomer-sequestering protein that inhibits actin polymerization, further increased the number of filopodia-like protrusions and decreased the number of spines in EB3 knockdown cells. The link between EB3 and the actin cytoskeleton appears to be p140Cap, a Src-binding protein that inhibits Src kinase activity. It was proposed that EB3-MT delivery of p140Cap to spines enables the inhibition of Src kinase phosphorylation of cortactin molecules to increase actin polymerization for spine growth. It should be noted that MT regulation of actin polymerization has been observed in fibroblasts and in nerve growth cones [18]. It is thus exciting to speculate that similar MT-to-actin pathways may be conserved and could be utilized (with possible variations, though) for different neuronal motile activities associated with growth cone migration and synaptic plasticity.
UNSOLVED ISSUES, FUTURE DIRECTIONS, AND LINK TO DISEASES

It is clear that these recent studies have presented evidence to show that dynamic MTs enter dendritic spines and may regulate spine dynamics and morphology. The imaging work together with pharmacological and molecular manipulations of MT dynamics has also indicated a potential role for dynamic MTs in spine development and synaptic plasticity. However, it remains to be determined about the precise functions of the MTs exploring spines. It should be noted, however, that all the manipulations on MT dynamics described (either MT-drug treatment or EB3 knockdown) were performed globally to the entire cultures. As a result, MTs throughout the entire neurons, including those in dendritic shafts, as well as in the presynaptic terminals, could be affected by these manipulations. Such global manipulation of MT dynamics could affect MT-based cellular functions, for instance, vesicle delivery in dendrites and presynaptic terminals, to generate secondary effects on dendritic spines. A definite determination of a functional role for MTs in spines requires spine-restricted manipulation of MT dynamics, such as the use of local photoactivation of caged MT-drugs (e.g. caged taxol) [24] or chromophore-assisted laser inactivation of MT components [48].

While the present evidence suggests that MTs in spines may act on the actin cytoskeleton for changes of spine shape, other potential functions for MTs in spine dynamics and synaptic plasticity remain to be explored (Fig. 1). One legitimate guess is that MTs may be involved in delivery of vesicular components and cellular organelles that are essential for postsynaptic structure and function. For example, mitochondria were found in about 9% of spines and smooth ER is found in about half of the spines [49, 50]. Spine localization of mitochondria was shown to be activity-dependent. Both smooth ER and mitochondria are known to depend on MTs for their transport and cellular distribution, thus they could, in principle, be transported into spines by a MT-based mechanism. Furthermore, polyribosomes were found to be recruited into spines after LTP induction [51]. The transport of polyribosome components and target mRNAs could depend on MTs as well for their spine localization. Finally, MTs could be involved in vesicle trafficking of neurotransmitter receptors during synaptic plasticity. In support of this possibility, it was reported that a labile component of AMPA receptor-mediated synaptic transmission depends on MT motors [52]. Future experiments that employ spatiotemporally-restricted manipulation of MT components at the single spine level, in combination with high-resolution imaging of synaptic changes, could potentially provide the answers to these important questions.

A role for MTs in spine development and plasticity could open up a new window for our understanding of the molecular and cellular mechanisms underlying several brain disorders. Given that many brain disorders are associated with abnormal spine morphology or density, it would be interesting to see if MTs are involved in defective neuronal connections. Indeed, defects in MT associated proteins have been linked to several brain disorders. For example, the fragile X mental retardation protein (FMRP) regulates MAP1b mRNA translation and its protein level, which is important for stabilizing MT networks [53]. Lack of FMRP has been shown to

**Fig. (1).** A schematic diagram illustrating potential functions of microtubules in dendritic spines. In addition to the proposed MT regulation of actin cytoskeleton through p140Cap and Src kinases on cortactin, MTs may also be involved in delivering membraneous organelles (e.g. mitochondria and receptor-containing vesicles), as well as ribosome/RNA complexes, to the dendritic spine. It is likely that MTs and the actin cytoskeleton cooperate in the delivery of these cargos into spines and in the regulation of spine structure and function.
result in filopodia-like immature spines and altered synaptic plasticity in fragile X-syndrome, possibly through its regulation of MAP1B translation. Disrupted-In-Schizophrenia 1 (DISC1) is a susceptibility gene for schizophrenia and bipolar disorder and it was reported that some schizophrenia patients exhibit low densities of dendritic spines in frontal and temporal cortex [54]. Recently, DISC1 protein was found to accelerate dendritic development and spine formation in adult newborn neurons [55]. Immuno-EM studies showed that DISC1 is enriched in spines and PSD, and some appeared to associate with MT structures [56, 57]. DISC1 also interacts with light chain 2 of MAP1A [58]. Although most reports studying the interaction of DISC1 and the MT structures focused on centrosome, there is a potential interplay between DISC1 and MTs in dendritic structure to regulate spine development. Finally, Williams syndrome is a developmental disorder caused by hemizygous deletion of approximately 28 genes on chromosome 7 [59]. The mild mental retardation of Williams syndrome patients is thought to be linked to two genes encoding proteins that regulate the cytoskeleton: LIM kinase 1, an upstream regulator of actin dynamics and CLIP-115, a MT +/− protein. Deletion of either of these two led to neurodevelopmental abnormalities in transgenic mice [60, 61]. LIMK1 knockout mice also exhibit immature spine morphology and enhanced LTP, suggesting that both actin and MT cytoskeleton are important for spine development and maturation.

In conclusion, these recent studies have added important evidence supporting the involvement of MTs in spine formation, dynamics, and plasticity that is associated with synaptic development and plasticity. Hopefully, future studies will provide a better understanding of MT functions in dendritic spines, which will enable us to dissect the molecular and cellular mechanisms underlying normal neural development and neurological disorders.

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