Deceivingly dynamic: Learning-dependent changes in stathmin and microtubules

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Abstract

Microtubules, one of the major cytoskeletal structures, were previously considered stable and only indirectly involved in synaptic structure and function in mature neurons. However, recent evidence demonstrates that microtubules are dynamic and have an important role in synaptic structure, synaptic plasticity, and memory. In particular, learning induces changes in microtubule turnover and stability, and pharmacological manipulation of microtubule dynamics alters synaptic plasticity and long-term memory. These learning-induced changes in microtubules are controlled by the phosphoprotein stathmin, whose only known cellular activity is to negatively regulate microtubule formation. During the first eight hours following learning, changes in the phosphorylation of stathmin go through two phases causing biphasic shifts in microtubules stability/instability. These shifts, in turn, regulate memory formation by controlling in the second phase synaptic transport of the GluA2 subunit of AMPA receptors. Improper regulation of stathmin and microtubule dynamics has been observed in aged animals and in patients with Alzheimer's disease and depression. Thus, recent work on stathmin and microtubules has identified new molecular players in the early stages of memory encoding.

1. Introduction

An organism's success and survival are critically dependent on its ability to process and transmit information related to a certain stimulus or threat in the environment. Therefore, it is crucial to unravel activity-dependent intracellular processes, as they provide the means for

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neural circuits to control adaptive behaviors. Increasing evidence demonstrates that extracellular stimuli initiate a chain of intracellular events with temporal patterns and signaling dynamics, which reflect the identity and intensity of information received from the environment (Behar and Hoffmann, 2010; Purvis and Lahav, 2013). For neurons, propagating intracellular signals with temporal patterns and signaling dynamics is especially challenging. Signals often travel long distances along neuronal projections, which are required for communication between the synapse and neuronal cell body and nucleus. This results in the strengthening of connections between synapses and thus, memory formation (Mayford et al., 2012).

Among many mechanisms mediating synaptic transport (Ch'ng and Martin, 2011), cytoskeletal structures – actin filaments and microtubules – play a major role in motor-driven import and export (Bredt and Nicoll, 2003; Hirokawa et al., 2010; Wenthold et al., 2003). Known to be dynamic and present in dendritic spines, actin filaments are essential in synaptic function and memory formation (Hotulainen and Hoogenraad, 2010; Tada and Sheng, 2006). In contrast, the role of microtubules, and, in particular, microtubule dynamics, in synaptic function and memory is less clear. Microtubule dynamics is a process involving a constant shift between microtubule stability and instability. There is a clearly established role for dynamic microtubules in cell division, axonal pathfinding during development, and axonal regeneration (Conde and Caceres, 2009). In contrast, microtubules in mature neurons are generally viewed as stable cytoskeletal structures present in dendritic shafts, but not in dendritic spines. Recently, work in hippocampal and cortical primary neuronal cell cultures has shown that microtubules, like actin filaments, can also be dynamically regulated during neuronal activity, move from the dendritic shaft to dendritic spines and be directly involved in synaptic structure and function (Gu et al., 2008; Jaworski et al., 2009; Kapitein et al., 2011; Merriam et al., 2011; Mitsuyama et al., 2008; Penzes et al., 2009). Thus, it is likely that microtubule dynamics in the dendritic spines and surrounding areas play important roles in synaptic plasticity and memory formation. Indeed, recent work in animals has begun to show that microtubule dynamics at synaptic sites are controlled by activity and learning, and that these changes are critical for long-term potentiation (LTP) and memory (Fanara et al., 2010; Uchida et al., 2014).

Here, we will summarize recent progress in the studies of the role of microtubules in synaptic structure, synaptic function and memory formation. In particular, we will focus on some recent findings that link activity-dependent changes in microtubule stability/instability and activity of stathmin, a negative regulator of microtubule formation.

2. Post-translational modifications (PTMs) of tubulin

Microtubules are composed of heterodimers of α- and β-tubulins. They are heterogeneous in length and highly dynamic *in vitro* and *in vivo*, undergoing cycles of polymerization and rapid depolymerization. Microtubule dynamics and function are highly controlled by the intrinsic GTPase activity of tubulins as well as various PTMs, such as tyrosination, detyrosination, acetylation, 2 modification, glutamylation, glycylation, palmitoylation, and phosphorylation (Fukushima et al., 2009; Janke and Bulinski, 2011; Yu et al., 2015). Some of these PTMs occur both on α-tubulin and β-tubulin. Most of tubulin PTMs happen on the

C-terminal tails that are critical for interaction with various molecular motors and microtubule-associated proteins (Yu et al., 2015). Modifications on the C-terminal tails include detyrosination/tyrosination of α-tubulin, the removal of the penultimate glutamate of α-tubulin (forming 2-tubulin), and glutamylation and glycylation of α- and β-tubulin tails (Yu et al., 2015). Although PTMs of tubulin have been known for more than 40 years, we are only now starting to understand their roles, as they emerge as crucial controllers of microtubule properties, defining microtubule diverse cellular functions in various biological systems.

In this review we will focus mostly on the tyrosination/tyrosination of tubulin because recent work has shown its critical role in memory formation (Uchida et al., 2014). α-tubulin undergoes the tyrosination/detyrosination cycle. It is initiated by the removal of a Tyr functional group (detyrosination). Re-addition of Tyr (tyrosination) then reverses the modification and returns tubulin to its nascent state. All tubulins, except for α4A- and α8-tubulin, possess a Tyr residue at the C-terminus immediately after translation, which is a marker of newly generated unstable microtubules (Khawaja et al., 1988; Paturle-Lafanechere et al., 1994). After assembly, tyrosinated tubulin in microtubules is detyrosinated. Detyrosinated tubulin is present in stable, long-lived microtubules (Khawaja et al., 1988; Paturle-Lafanechere et al., 1994). As discussed below, detyrosination and tyrosination of tubulin are highly regulated by learning (Table 1).

Acetylation of Lys40 on α-tubulin (L'Hernault and Rosenbaum, 1985), similar to the detyrosination, takes place on the microtubule polymer (Maruta et al., 1986). Acetylated tubulin exists in stable, long-lived microtubules, although acetylation does not seem to cause microtubule stabilization (Palazzo et al., 2003). Although the role of tubulin PTMs in microtubule dynamics is not well understood, it is important to mention that some of the critical microtubule functions are dependent on microtubule dynamics, which in turn are controlled by PTMs of tubulin.

3. Activity-dependent changes in microtubules in dendritic spines

Dendritic spines are the major sites of excitatory synaptic input in the mammalian central nervous system. Their morphological changes are believed to be the basis of learning and memory (Kandel, 2001). Dendritic spines of neurons are enriched with actin filaments, and both spine structure and function are regulated by actin (Hotulainen and Hoogenraad, 2010; Tada and Sheng, 2006). Dynamics changes in actin filaments are important for structural modification in neurons. In contrast to what is known about actin filaments, microtubules were generally thought to be stable in mature neurons and not present in the spines. However, recent studies have shown that microtubules enter the spines under physiological conditions in cultured hippocampal and cortical primary neurons (Gu et al., 2008; Hu et al., 2008; Jaworski et al., 2009) and in the hippocampal slices after tetanic stimulation (Mitsuyama et al., 2008) (Table 1). In addition, tubulin posttranslational modifications such as tyrosination and acetylation are associated with neuronal activity (Table 1). Using time-lapse total internal reflection fluorescence microscopy in cultured hippocampal and cortical neurons, Dent and colleagues have shown that dynamic microtubules invade dendritic spines (Hu et al., 2008). They showed that treatment with BDNF increases tyrosinated-tubulin in

spines, whereas paclitaxel, a microtubule-stabilizing drug, abolishes this effect. This result indicates that BDNF increases microtubule invasion of dendritic spines, and that these invasions are dependent on microtubule dynamics. In addition, by imaging microtubule dynamics, they found that 10% of dendritic protrusions are targeted by microtubules in an hour. Interestingly, they found that microtubule invasion occurs in very mature hippocampal neurons (DIV63 or 63 days in vitro), suggesting that microtubule dynamics in spines appears to be an ongoing process throughout life of an organism. Zheng and colleagues also reported that microtubules can be present in spines of cultured hippocampal neurons using immunocytochemistry (Gu et al., 2008). They showed that a knockdown of EB3, a microtubule end-binding protein, leads to a decrease in the number of dendritic spines. Furthermore, they showed that blocking microtubule dynamics with nocodazole inhibits the BDNF-induced increase in the number of spines in neuronal cultures. This was confirmed by another study, which also found microtubule invasions in hippocampal dendritic spines (Jaworski et al., 2009). Importantly, microtubule entry into spines was increased 3-fold after transient stimulation of cultured hippocampal neurons with potassium chloride, and this increase was blocked by treatment with tetrodotoxin, indicating that microtubule dynamics in neurons are changing in an action potential-dependent manner (Hu et al., 2008). Finally, it was shown that the microtubule entry into dendritic spines is synaptic activity-dependent (Mitsuyama et al., 2008). These studies clearly demonstrate invasion of microtubules in dendritic spines in an activity-dependent manner and provide the first important clues regarding the role of microtubule dynamics in post-mitotic neurons.

4. Microtubule-mediated intracellular transport

In neurons, the transport between the cell body and synapses is fundamental for synaptic function and for memory. Microtubules play a critical role in transport of various molecules and organelles, both pre- and post-synaptically. Importantly, PTMs of tubulin are associated with intracellular transport through the regulation of microtubule stability and motor protein activity. This suggests that activity-dependent changes in PTMs of tubulin might control synaptic transport, thereby mediating synaptic plasticity and memory formation. Molecular motors from the kinesin, dynein, and myosin superfamilies are normally involved in transport of various cargoes, and two of those, kinesins and dyneins, are utilized by microtubules (Hirokawa et al., 2010). Microtubule stability has been reported to affect the activity of motor proteins, thereby influencing intracellular transport.

4.1. Role of KIF in dendritic transport

In dendrites, cargo is transported by kinesin superfamily of proteins (KIFs) (Hirokawa et al., 2010). Some of the cargo includes the N-methyl-D-aspartate receptors (NMDARs), which are critical for synaptic plasticity, learning and memory. KIF17 is a motor protein that transports GluN2B, a subunit of NMDAR-containing vesicles (Hirokawa et al., 2010). The disruption of the *kif17* gene inhibits GluN2B transport, resulting in a loss of synaptic GluN2B (Yin et al., 2011). Critically, NMDA receptor-mediated synaptic currents, early and late long-term potentiation (LTP), long-term depression (LTD), and CREB responses are attenuated in KIF17 knockout mice, which is consistent with hippocampus-dependent memory impairment (Yin et al., 2011). Another kinesin, KIF5, associates with GRIP, a

scaffolding protein that binds to α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs) and transports them in dendrites (Setou et al., 2002). In addition, microtubule motor function of KIF5 is required for AMPAR-mediated synaptic transmission (Hoerndli et al., 2013). Moreover, γ-aminobutyric acid receptors (GABARs), a critical component of the inhibitory system in the brain, are transported by KIF5 via huntingtin-associated protein 1 (HAP1) (Twelvetrees et al., 2010). In conditional KIF5A knockout mice, in which KIF5A protein is postnatally deleted in neurons, impaired GABA(A) receptor-mediated synaptic transmission leads to an epileptic phenotype, thus demonstrating that KIF5A may regulate inhibitory transmission (Nakajima et al., 2012).

4.2. Microtubule stability influences motor proteins activity

Regulating the activity of molecular motors via microtubule PTMs provides a mechanism for transporting cargo to the corresponding synaptic sites (Janke and Bulinski, 2011). Tubulin detyrosination has been shown to regulate the binding and motor activity of the KIF5 to microtubules (Dunn et al., 2008; Konishi and Setou, 2009; Liao and Gundersen, 1998). Tubulin detyrosination increases the affinity of KIF5 for microtubules ~2.8-fold (Dunn et al., 2008; Konishi and Setou, 2009; Liao and Gundersen, 1998) affecting long-distance transport processes. Although all PTMs of tubulin are found on neuronal microtubules (Janke and Bulinski, 2011), their role in synaptic plasticity and memory has remained unclear. Knockout and transgenic approaches to study individual microtubule-modifying enzymes will provide direct evidence for their function *in vivo*.

5. Microtubules in synaptic plasticity and memory formation

Microtubules are critical for synaptic transport, but how dynamically changing microtubules are involved in learning and memory has remained unclear. As LTP is one of the forms of synaptic plasticity and a leading model of the cellular mechanisms responsible for memory formation (Bliss and Lomo, 1973; Kandel et al., 2014), it is important to note that drugs inhibiting microtubule dynamics interfere with LTP. For example, applying a microtubule-stabilizing drug, paclitaxel, leads to LTP deficits in the cortico-amygdala pathway in mouse brain slices (Shumyatsky et al., 2005). Also, an inhibitor of microtubule dynamics, nocodazole, decreases mossy fiber LTP (Barnes et al., 2010) and Schaffer collateral LTP (Jaworski et al., 2009). Since drug interfering with microtubule dynamics affect LTP, it is likely that microtubules are also involved in memory formation.

Indeed, recent evidence in live animals has revealed a previously unknown feature of microtubules: learning induces biphasic shifts in microtubule stability in the dentate gyrus of hippocampus (Uchida et al., 2014). The level of detyrosinated tubulin (marker of stable microtubules) was changed in a biphasic manner, first decreasing within 30–60 min following contextual fear conditioning and then increasing at the time point of 8 h following conditioning, whereas tyrosinated tubulin (marker of unstable microtubules) levels were changed in the opposite direction (Table 1). This suggests that microtubules become unstable 0.5–1 h and hyperstable 8 h following learning (Figure 1). Exposure to context only or immediate shock led to a reduction of the detyrosinated tubulin and an increase in tyrosinated tubulin 0.5 h, but not 8 h, following the exposure (Uchida et al., 2014). These

results suggest that the increase in microtubule stability at 8 h is specific to associative learning.

Pharmacological manipulation of hippocampal microtubule activity further supports an important role of biphasic changes in microtubule stability in memory formation. Intra-hippocampal injection of nocodazole 8 h following contextual fear conditioning causes a decrease in long-term memory. By contrast, infusion of paclitaxel, a microtubule stabilizer, increases memory (Uchida et al., 2014). These pharmacological experiments demonstrate that memory can be enhanced or disrupted by changing microtubule hyperstability 8 h following learning. They also suggest that during this time period microtubule-dependent intracellular processes involved in memory consolidation are sensitive to manipulation (see also Section 9).

The learning-induced destabilization of microtubules during the early phase is also important for memory formation. Intra-hippocampal injection of paclitaxel immediately after training disrupts long-term memory in contextual fear conditioning (Uchida et al., 2014). This suggests that the early phase has a significant role in associative memory formation. To examine the role of the early phase of microtubule changes in memory, further work needs to be performed (also see Chapter 8).

A study by Fanara et al. (2010) also showed a role of microtubules in neuronal plasticity and memory. Using stable isotope labeling to measure the turnover of tubulin in defined microtubule populations in the mouse brain, they found increased turnover of hippocampal MAP2-associated and cold-stable microtubules following contextual fear conditioning. Nocodazole infusion into the brain prevented learning-induced increase in MAP2-associated microtubules and reduced contextual fear memory. In addition, nocodazole infusion blocked learning-dependent enhancement of spine density in the hippocampus, demonstrating a link between spine structural plasticity and microtubule dynamics *in vivo*.

6. Stathmin, a microtubule-destabilizing protein

Although several lines of evidence have shown that microtubule dynamics are important for synaptic plasticity and memory formation, the molecular mechanisms underlying learning-mediated control of microtubule dynamics are largely unknown. Proteins that act as both microtubule stabilizers and destabilizers have been identified in neurons. These include microtubule-interacting proteins, microtubule-associated proteins, microtubule-destabilizing proteins, microtubule-severing proteins, microtubule plus-end tracking proteins (+TIPs), and motor proteins (Akhmanova and Steinmetz, 2008; Conde and Caceres, 2009; Dent and Gertler, 2003; Fukushima et al., 2009; Janke and Bulinski, 2011; Rodriguez et al., 2003). This review will focus on a microtubule destabilizer stathmin/oncoprotein 18 (also known as stathmin 1). Stathmin (stathmin 1) is a member of the stathmin family of phosphoproteins, which also includes superior cervical ganglia neural-specific 10 protein (SCG10; stathmin 2), SCG10-like protein (SCLIP; stathmin 3), and RB3 (stathmin 4). Among them, stathmin is the most abundantly expressed in the nervous system (Chauvin and Sobel, 2015). In addition, stathmin is highly expressed in the rodent brain during development and in adulthood, whereas the expression of SCG10, SCLIP and RB3 gradually decrease during

development and stay low or at not detectable level in the adult rodent brain (Boekhoorn et al., 2014). Thus, stathmin (stathmin 1) may be the only member of the stathmin family controlling microtubule dynamics in the adult brain.

Stathmin has four Ser residues (Ser16, Ser25, Ser38 and Ser63), all of which can be phosphorylated (Di Paolo et al., 1997; Larsson et al., 1997). Unphosphorylated or hypophosphorylated stathmin binds tubulin heterodimers, preventing microtubule assembly. After its phosphorylation, stathmin releases tubulin dimers, allowing microtubules to be formed. Although phosphorylation of stathmin at all Ser residues is important for inactivation of its depolymerization activity (Lawler, 1998; Melander Gradin et al., 1997), phosphorylation at Ser16 strongly reduces the binding affinity of stathmin to tubulin heterodimers (Di Paolo et al., 1997; Larsson et al., 1997; Manna et al., 2009). It should be noted that Ser16 can be phosphorylated by cAMP-dependent protein kinase A (PKA) and Ca^{2+}/Calmodulin-dependent kinases II and IV (CaMKII/IV) (Beretta et al., 1993; le Gouvello et al., 1998; Marklund et al., 1994), which are critically involved in brain function and in memory (Abel et al., 1997; Fukushima et al., 2008; Kang et al., 2001; Malleret et al., 2010; Mayford et al., 1996; Silva et al., 1992). Thus, these kinases may regulate memory formation by phosphorylating stathmin at Ser16 and thus controlling microtubule stability. Other three Ser residues are also likely to be involved in microtubule-depolymerizing activity of stathmin following behavior or brain activity.

7. Role of stathmin in synaptic plasticity and behavior

The first evidence that members of the stathmin family are involved in activity-dependent processes in the adult brain came with the demonstration of RB3 protein being induced in the dentate gyrus granule layer of the hippocampus by electrically induced seizure activity or by stimuli that lead to LTP (Beilharz et al., 1998). While both basic synaptic transmission and NMDAR function are normal, LTP is deficient in the cortico-amygdala pathway in stathmin knockout mice (Shumyatsky et al., 2005). In addition, mice overexpressing the unphosphorylatable Stathmin4A mutant have deficiency in perforant path-dentate gyrus LTP (Uchida et al., 2014).

Other studies also have shown that stathmin may play a role in neuronal plasticity (Table 2). Stimulation of cultured primary neurons with BDNF increases phosphorylation of stathmin (Cardinaux et al., 1997). In addition, phosphorylation of stathmin is induced by treatment with dopamine or forskolin (Cardinaux et al., 1997). Given that BDNF and forskolin are widely known to influence neuronal plasticity and memory, phosphorylation of stathmin might be an underlying mechanism of synaptic plasticity and memory (see Sections 8 and 9).

Accumulated evidence has indicated which brain areas and behaviors are dependent on stathmin function. Stathmin was first found to be functionally important for cortico-amygdala LTP and behaviors dependent on the basolateral amygdala-associated neural circuitry, including the anatomical pathways relaying both conditioned and unconditioned stimuli information to the basolateral amygdala (Shumyatsky et al., 2005). This expression pattern allows stathmin to control innate and learned fear. In addition, stathmin knockout

mice display enhanced extinction of cued fear conditioning, which was accompanied by an increase in neural activity in the prefrontal cortex and dentate gyrus with a simultaneous decrease in amygdala activity (Martel et al., 2012). Stathmin knockout mice also show an increase in social interactions but a decrease in social recognition memory and affiliative maternal care, which were associated with stathmin expression in the basolateral amygdala (Martel et al., 2008). Stathmin transgenic mice expressing Stat4A, an unphosphorylatable stathmin mutant, show reduced contextual fear memory (Uchida et al., 2014), suggesting that stathmin phosphorylation plays an important role in brain function.

8. Biphasic changes in stathmin activity following learning

Stathmin function is regulated by various stimuli in cultured neurons, but what about its role *in vivo* following learning? A recent study has found that learning biphasically regulates stathmin phosphorylation and tubulin-binding activity at synaptic sites (Table 2) (Uchida et al., 2014). During the first phase, phosphorylation of synaptosomal stathmin at the Ser16, Ser25 and Ser38 sites was rapidly decreased 15–60 min following training in fear conditioning. During the second phase – 8 h following fear conditioning – phosphorylation of synaptosomal stathmin at Ser16 and Ser38 sites was increased. There was no effect of either the context only or the immediate shock on stathmin phosphorylation at Ser16, suggesting a specific role of Ser16 phosphorylation in memory formation. Importantly, stathmin-tubulin binding was increased 30 min following 8 h after training and it was decreased 8 h following training. In mice exposed to context only, stathmin binding to α-tubulin was increased 30 min following exposure, but not after 8 h,. These data suggest that during associative learning, stathmin may regulate microtubule stability by changing its affinity to α-tubulin.

It should be noted that during both the early and late phases, shifts in learning-dependent microtubule stability are accompanied by parallel changes in stathmin phosphorylation and in the microtubule-destabilizing activity of stathmin (Figure 1), suggesting that stathmin can directly control the shifts in microtubule stability during memory encoding and can be critically involved in memory formation. Supporting this idea, both gain- and loss-of-function stathmin mutations disrupt learning-dependent microtubule dynamics and memory in contextual fear conditioning (Uchida et al., 2014). Changes in stathmin activity and microtubule stability are seen in synaptosomal fractions, but not in whole-cell extracts. Because these changes accompany deficits in synaptic plasticity and contextual fear memory, the synaptosomal localization of the changes suggests the importance of stathmin-microtubule interactions at the synaptic sites for memory processing.

Microtubule-depolymerizing activity of stathmin (measured by its phosphorylation status) is induced 15 min after learning and then followed by an increase in microtubule instability 15 min later (30 min after learning; Figure 1). Initial microtubule disassembly caused by a decrease in stathmin phosphorylation, and thus an increase in stathmin activity, is likely to be followed by a subsequent, new microtubule assembly, since the level of tyrosinated tubulin, a marker of labile newly generated microtubules, is increased. Exposure to only context or shock dephosphorylates stathmin to the extent that is not as pronounced as that after context-US exposure, which represents associative memory. However, the changes

during the early phase are seen both after context-US association and after exposure to context or immediate shock. Thus, they might reflect molecular events related to attention or arousal, which is somewhat similar to induction of expression observed in immediate-early genes (Kubik et al., 2007). It is plausible that the early phase is related to an arousal/ attention or memory trace, which can be transferred to a long-lasting form of memory if it becomes biologically relevant (for example after being associated with a shock).

Learning-induced changes in stathmin phosphorylation seem to directly regulate its microtubule-destabilizing activity and thus microtubule stability. This is illustrated by the dysregulation of changes in microtubule stability in Stat4A transgenic mice, overexpressing the unphosphorylatable Stathmin4A mutant. While in wild-type mice, an increase in detyrosinated tubulin normally occurs 8 h following contextual fear conditioning, in Stat4A mice, it was disrupted, suggesting improper regulation of microtubule stability (Uchida et al., 2014). This result indicates that phosphorylation of stathmin is essential for microtubule hyperstability following learning. In addition, stathmin knockout mice showed suppression of changes in microtubule stability during the early and late phase and were deficient in contextual fear memory as tested 24 h after training (Uchida et al., 2014). These data indicate that in the absence of stathmin, both early instability and late hyperstability in microtubules are deficient following learning, leading to deficits in memory formation.

The changes in stathmin phosphorylation within the first eight hours following learning described above do not involve changes in total amount of stathmin protein. However, it appears that the total levels of stathmin mRNA and protein in the dentate gyrus of the rat hippocampus are increased 48 h following contextual fear conditioning (Federighi et al., 2013). This finding suggests that following learning stathmin may be involved in other intracellular processes in addition to the synapse-specific events (Uchida et al., 2014).

9. How do stathmin-mediated microtubule dynamics affect memory process?

9.1. Stathmin-mediated control of microtubule stability is associated with intracellular transport

As mentioned earlier, increased stability of microtubules and hyper-phosphorylation of stathmin during the late phase following learning are specifically observed in the memory process. This fact raises the possibility that increased detyrosination of tubulin during the late phase may influence motor protein function, which in turn affect memory formation (Figure 2). Indeed, in hippocampal primary cell cultures, tubulin detyrosination enhances the motor activity of KIF5 and its binding to microtubules (Konishi and Setou, 2009). In agreement with this, there is an increase in the interaction between KIF5 and α-tubulin 8 h following learning (Uchida et al., 2014). In addition, learning induces KIF5-GluA2 interactions and increased GluA2 levels in the synaptosomal and microtubule fractions during the late phase (Uchida et al., 2014), which is consistent with the report that the dendritic GluA2 is transported by KIF5 (Setou et al., 2002). Confirming these interactions, Stat4A transgenic mice expressing the constitutively active Stathmin4A mutant, show a lack of dendritic transport of GluA2 with KIF5 following learning (Uchida et al., 2014). The

finding that KIF5-GluA2 binding is regulated by stathmin-microtubule interactions is in agreement with work showing that the gain-of-function stathmin mutation leads to a decrease in KIF5 levels in the cerebellar dendrites (Ohkawa et al., 2007), further supporting the notion that stathmin-mediated microtubule dynamics influence KIF5-driven intracellular transport. The functional importance of the change in microtubule-mediated GluA2 synaptic transport is confirmed by the ability of the TAT-GluA2$_{3Y}$ peptide, a blocker of GluA2 endocytosis, to rescue contextual fear memory when injected into the dentate gyrus area of Stat4A mice and is in agreement with the role of AMPAR transport in synaptic plasticity (Correia et al., 2008) and contextual memory (Matsuo et al., 2008; Mitsushima et al., 2011; Rao-Ruiz et al., 2011). This evidence suggests an important role for stathmin-mediated dendritic transport of the GluA2 along microtubules in synaptic plasticity and memory formation.

9.2. Possible role of stathmin and microtubules in tagging of synapses

Accumulated evidence suggests that individual synapses are "tagged" by previous synaptic input, leading them to be selectively recognized as targets for recruitment of new synaptic molecules (reviewed in Martin and Kosik, 2002). Kinesin-mediated transport of proteins, mRNA, or organelles that are involved in synaptic development and plasticity seem to be associated with synaptic tagging (Dent et al., 2011). Thus, it is plausible that learning-associated changes in microtubule dynamics at synaptic sites are involved in the "tagging" of the synapses for further modifications by GluA2. It would be interesting, in the future, to investigate whether the same synaptic sites undergo changes in microtubule stability during the early and late phases following learning. Further studies are required to understand the role of microtubule dynamics in synaptic homeostasis in the dentate gyrus, as well as other brain regions.

Learning-dependent regulation of dynamic microtubules by stathmin at synaptic sites in the adult mammalian brain plays an important role in memory formation (Figure 2). Learning induces changes in phosphorylation of stathmin and its ability to bind tubulin, which in turn leads to shifts in microtubule dynamics and regulates microtubule-mediated intracellular localization of the GluA2 subunit of AMPARs. These intercellular changes control synaptic plasticity and memory formation. Thus, learning-dependent control of microtubule dynamics by stathmin represents a new signaling pathway crucially involved in memory formation.

10. The biphasic shifts in stathmin and microtubules confirm the hypothesis of multiple waves of neuronal plasticity during memory consolidation

It is interesting to note that several other cellular processes show biphasic changes following learning. For example, training in the water maze leads to biphasic changes in phosphorylation of cAMP-response element-binding protein (CREB) in the CA1 area of the hippocampus, with two different peaks occurring 15 min and 8 h post-acquisition (Porte et al., 2011). Also, in the CA1, training in tone-shock fear conditioning leads to a monophasic increase in phosphorylation (15 min following training) of extracellular signal-regulated kinase 1/2 (ERK) and CREB, while context-shock conditioning leads to a biphasic increase

in ERK/CREB phosphorylation (Trifilieff et al., 2006). The monophasic change in ERK/
CREB phosphorylation in CA1 following tone-shock is somewhat reminiscent to the early
phase of the changes in stathmin and microtubules in the dentate gyrus following exposure
to tone or shock only. The immediate-early gene coding for activity-regulated cytoskeleton-
associated protein (*Arc*), which regulates AMPAR trafficking, shows a biphasic increase in
protein expression (0.5-1 and 8 h) in the hippocampus following spatial exploration of a
novel environment (Ramirez-Amaya et al., 2005). In another study, hippocampal Arc levels
after contextual fear conditioning increase in a biphasic manner immediately and 12 h after
conditioning, and selectively blocking late Arc expression interferes with persistence, but
not formation, of contextual fear memory (Nakayama et al., 2015). Similar biphasic changes
in gene transcription- or protein translation-related processes have been observed in other
studies (Bourtchouladze et al., 1998; Izquierdo et al., 2002; Martel et al., 2007; Stanciu et
al., 2001; Swank and Sweatt, 2001; Trifilieff et al., 2007). Thus, the biphasic shift in
stathmin activity and microtubule stability resembles previous observations of biphasic
changes following exploration or learning, which led to the suggestion that multiple waves
of neuronal plasticity are involved in memory consolidation (Rose, 2000). Therefore, there
is now evidence for multiple time windows during memory formation. These waves and
their timing seem to depend on the exact task used, the brain region studied and the
molecular process being examined. Another general concern with time windows is the
potential for circadian effects. For example, large time-of-day effects on gene expression in
the hippocampus were found, suggesting that some of the reported changes may be circadian
regulated (Peixoto et al., 2015). So, one issue is doing appropriate time of day controls. This
might also relate to the role of sleep in memory.

Given all these concerns, biphasic changes in stathmin and microtubules are unique in a
sense that the first phase leads to instability and the second phase leads to hyperstability.
Most of the other biphasic transcription/translation processes show an increase or decrease
in both phases.

11. Implication of stathmin and microtubule in neuropsychiatric disorders

All previous sections in this review discussed the role of stathmin and microtubules in
regulating neuronal function in the healthy brain. But is there any evidence implicating
stathmin and microtubules in brain dysfunction? We will review several examples of these
molecules being involved in mental states and discuss how stathmin, by controlling
microtubule dynamics, may be responsible for the pathophysiology of cognitive dysfunction
in the adult brain, as well as in age-related disorders.

11.1. Age-related memory loss and Alzheimer's disease

Stathmin-microtubule interactions seem to be one of possible mechanisms underlying age-
dependent memory loss. Interestingly, stathmin expression in the brain is decreased with
aging (Saetre et al., 2011) and this decrease is accelerated in Alzheimer's disease (Hayashi
et al., 2006; Jin et al., 1996; Saetre et al., 2011), linking stathmin to aging processes.

The dentate gyrus has been implicated in normal aging in humans and primates (Burke and
Barnes, 2006; Small et al., 2004). Also, reduced levels of the synaptic GluA2 in the dentate

gyrus were associated with memory loss in aged monkeys (Hara et al., 2012). In support of the hypothesis that stathmin and microtubules are involved in aged-dependent memory loss, aged wild-type mice show reduced stathmin levels as well as reduced levels of GluA2 at the synaptic sites and in microtubule fractions of the dentate gyrus (Uchida et al., 2014). Furthermore, blocking the GluA2 endocytosis reverses the deficits in contextual fear memory in aged mice, suggesting that the deficit in the GluA2 transport has a crucial role in age-dependent memory loss. This implicates stathmin and microtubules involvement in the age-related changes in GluA2 transport (Uchida et al., 2014). Since changes in stathmin-mediated microtubule dynamics influence GluA2 intracellular transport from the cell body to synaptic sites (Uchida et al., 2014), the stathmin-microtubule-GluA2 signaling pathway might be associated with age-dependent memory loss and might use a similar mechanism as that in young adult mice.

Other work also suggests a role for microtubules in memory and cognition. Paclitaxel-like drug Epothilone D (which crosses the blood-brain barrier) improves memory in an Alzheimer's disease mouse model when used in a very low dose. Phase I clinical trials with low doses of Epothilone D in Alzheimer's patients were initiated (Barten et al., 2012).

11.2 The role of stathmin and microtubules in cognitive impairments

Many signaling pathways involved in memory and cognition are also found to be disrupted in various mental disorders (Ebert and Greenberg, 2013; Pittenger and Duman, 2008). Thus, it is not surprising that growing evidence links dysfunction in the adult brain to stathmin and microtubules. Stathmin has been implicated in abnormal states of fear, anxiety, cognition, social behavior, and brain trauma in rodents and humans (Brocke et al., 2010; Ehlis et al., 2011; Elder et al., 2012; Martel et al., 2012; Martel et al., 2008; Shumyatsky et al., 2005), suggesting microtubule involvement in these processes. Common single nucleotide polymorphisms (SNPs) in the STMN1 gene significantly impacted fear and anxiety responses in humans, measured with the startle and cortisol stress response (Brocke et al., 2010). In another human study, carriers of the SNP rs182455 of STMN1 showed altered cognitive-affective processing with the effects more pronounced in females (Ehlis et al., 2011). In a rat model of mild traumatic brain injury (mTBI) that leads to post-traumatic stress disorder (PTSD), animals exposed to repetitive blast injury displayed elevated levels of stathmin in the amygdala (Elder et al., 2012). Finally, stathmin knockout mice show increased extinction of cued fear conditioning, which affects neuronal activity as measured with c-fos in the dentate gyrus, amygdala and prefrontal cortex (Martel et al., 2012).

Recent evidence also links depressive-like behaviors to cytoskeleton-related changes, both in actin filaments and microtubules (Wong et al., 2013). Chronic unpredictable stress, which is a laboratory model for studying depression, leads to changes in the ratio of tyrosinated tubulin and acetylated tubulin in rat hippocampus, suggesting changes in microtubule stability (Yang et al., 2009). Also, in a proteomics study, changes in tubulin have been reported in an animal model of depression (Piubelli et al., 2011). An increase in microtubule acetylation in the hippocampus is associated with decreased neuronal plasticity and dendritic retraction by chronic stress (Bianchi et al., 2003; Yang et al., 2009). Tubulin becomes less tyrosinated in a depression rat model (Bianchi et al., 2003), and there is an increase in

tyrosinated tubulin in sleep-deprived rats, indicating microtubule instability (Basheer et al., 2005).

In post-mortem hippocampus of depressed subjects there are changes in microtubule-associated proteins, MAP1A, MAP1B and MAP2, as well as in genes, which protein transport is dependent on microtubules, such as GluA1 and GluA3 subunits of AMPARs (Duric et al., 2013). As the mechanisms responsible for learning and depression overlap, it is intriguing that we reported learning-dependent synaptic accumulation of the GluA2 subunit and its motor protein, KIF5, to be deficient in transgenic mice expressing a constitutively active Stathmin4A mutant, which leads to deficits in microtubule dynamics (Uchida et al., 2014). Also, MAP2, which modulates microtubule dynamics, is proposed to be a target of drugs that stimulate tubulin assembly and act as antidepressants having advantages over selective serotonin re-uptake inhibitor fluoxetine (Bianchi and Baulieu, 2012).

In addition, recent evidence has suggested that deficits in stathmin and microtubules might be involved in schizophrenia and Huntington disease (Cao et al., 2013; Colin et al., 2008; Dompierre et al., 2007; Hayashi et al., 2006). Thus, stathmin and dynamic microtubules may represent a convergent pathway for different biological processes in health and disease of the nervous system. As a result of these similarities, changes in microtubule stability and/or improving microtubule dynamics may be a beneficial approach to treat memory deficits in aging, Alzheimer's disease, as well as establish axonal and synaptic connections in spinal cord injury and central nervous system lesions (Barten et al., 2012; Hellal et al., 2011; Witte et al., 2008). Thus, elucidating the molecular mechanisms of stathmin-microtubule interactions or downstream/upstream signaling of microtubule dynamics may help better understand the pathophysiology of neuropsychiatric disorders and may lead to better design of novel approaches for their treatment.

12. Conclusion

A recent finding demonstrates that learning-induced changes in stathmin-dependent dynamic microtubules regulate GluA2 synaptic intracellular transport and this process is crucial for memory formation (Uchida et al., 2014). Taken together, the above studies indicate that there are emerging roles for stathmin in microtubule stability, protein transport, synaptic plasticity and memory formation. Throughout this review we have focused on the stathmin-mediated regulation of microtubule dynamics in memory formation. However, it is still unclear how stathmin activity is controlled in response to synaptic input. Also, stathmin-interacting proteins, which regulate stathmin microtubule-destabilizing activity, are still not well-characterized. Finally, control of localization of the GluA2 subunit of AMPARs is likely not the only process controlled by stathmin-microtubule interactions following learning. Thus, it will be instructive to dissect further both upstream and downstream signaling in the stathmin-microtubules interactions to fully understand the role of this pathway in brain function.

Acknowledgments

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Highlights

- Learning induces early and late phases in stathmin activity and microtubule stability

- Contextual fear conditioning is controlled by dentate gyrus stathmin

- In the late phase stathmin and microtubules control synaptic localization of AMPARs

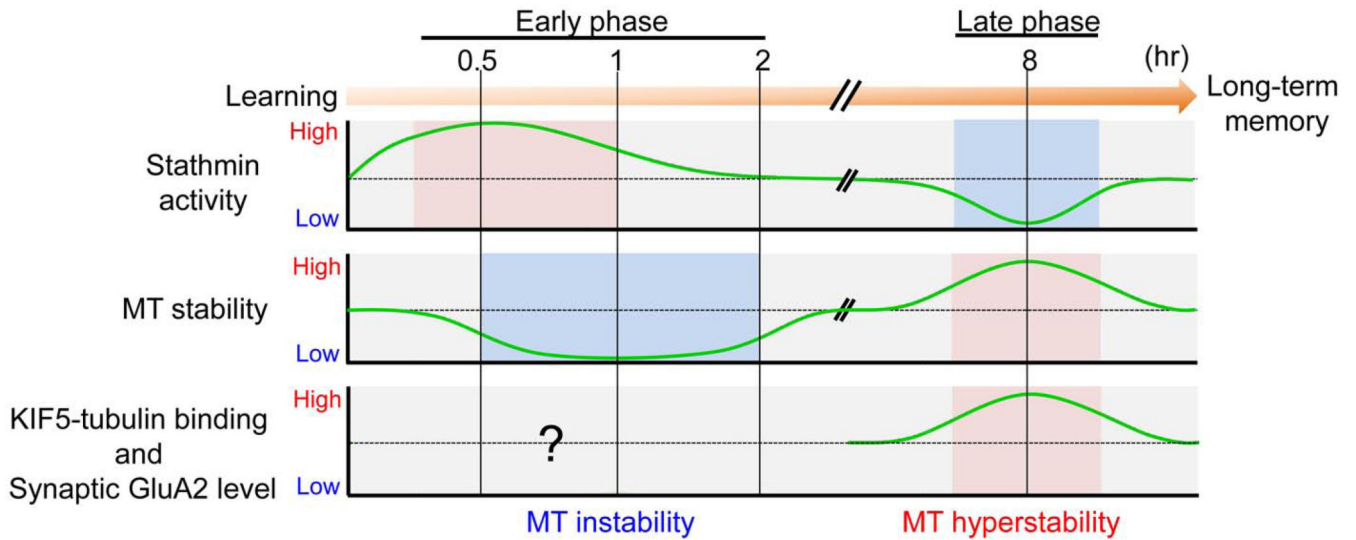- Aging and mental disorders may involve stathmin and microtubule changes

Figure 1. The early and late phases of microtubule stability and stathmin activity following learning

In the early phase, stathmin is dephosphorylated and binds to tubulin during the first 15-60 min following contextual fear conditioning. This leads to microtubule disassembly by activated stathmin. 0.5-2 h after learning, tyrosinated-tubulin-enriched microtubules (labile, newly generated microtubules) are formed. Although it is suggested that the activity-dependent dynamic microtubules (*eg*., tyrosinated tubulin) can entry into the spine in primary neuronal cell cultures (see reviews (Dent et al., 2011; Penzes et al., 2009)), it is still unclear whether this also occurs in the mouse brain. In the late phase (8 hours) after the learning, phosphorylation of stathmin is increased and the stathmin is dissociated from tubulin. Subsequently, the amount of detyrosinated-tubulin-enriched microtubules (stable microtubules) is increased. Tubulin detyrosination activates the transport of GluA2 subunit of AMPARs from the soma to synaptic sites by enhancing the binding of KIF5-GluA2 complex to microtubules.
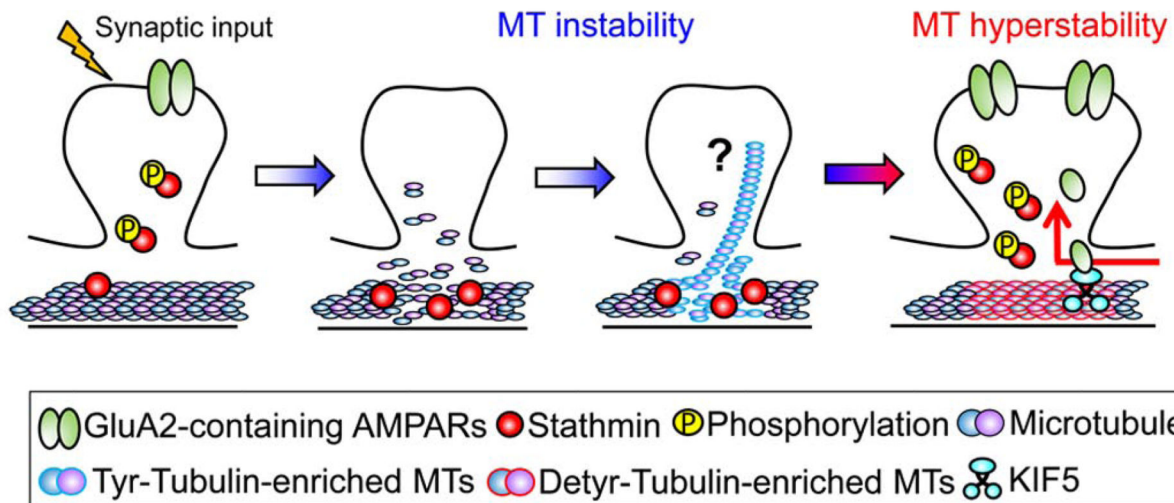
Figure 2. Proposed Model for the Role of Dynamic Microtubules in Memory Formation
During the early phase (see Figure 2), microtubules become unstable due to the increased binding of stathmin to the tubulin caused by the hypophosphorylation of stathmin. Microtubule instability during the early phase is important for memory formation because treatment with microtubule stabilizer drug paclitaxel disrupts long-term memory formation (see text for details). During the late phase, phosphorylation of stathmin is increased and tubulin is dissociated from stathmin. Subsequently, the amount of detyrosinated-tubulin-enriched microtubules (stable microtubules) is increased. Tubulin detyrosination activates the transport of the GluA2 subunit from the soma to synaptic sites by enhancing the binding of the KIF5-GluA2 complex to microtubules. The microtubule hyperstability during the late phase is also important for memory, since treatment with microtubule stabilizer drug paclitaxel or microtubule destabilizer drug nocodazole 8 h following learning enhances or reduces long-term memory, respectively.

Table 1

Brief summary of activity-dependent changes in microtubule stability in neuronal cells

Microtubule dynamics			
Targets	**System**	**Findings**	**References**
Tyrosinated tubulin	Primary neuron	Tyrosinated tubulin extended into dendritic protrusions. Potassium chloride treatment increases microtubule invasions of dendritic protrusions.	(Hu et al., 2008)
Tubulin EB3	Primary neuron	Microtubule invasions of dendritic protrusions. BDNF-induced increase of spine number is blocked by microtubule destabilizer (nocodazole) treatment.	(Gu et al., 2008)
EB3	Primary neuron	Microtubule invasions of dendritic protrusions.	(Jaworski et al., 2009)
Tubulin	Hippocampal slices	Electron microscopic analysis shows that there is redistribution of microtubules in dendritic spines following LTP induction.	(Mitsuyama et al., 2008)
Acetylated tubulin	Hippocampal slices	Potassium chloride or NMDA treatment enhances the level of acetylated tubulin.	(Pandey and Sharma, 2011)
Tyrosinated tubulin	Mouse dentate gyrus (contextual fear conditioning)	Tyrosinated tubulin increases 0.5-1 h and decreases 8 h following context-shock exposure. Increased tyrosinated tubulin is observed in mice received context only or immediate shock.	(Uchida et al., 2014)
Detyrosinated tubulin	Mouse dentate gyrus (contextual fear conditioning)	Detyrosinated tubulin decreases 0.5-1 h and increases 8 h following context-shock exposure. Decreased detyrosinated tubulin is observed in mice received context only or immediate shock.	(Uchida et al., 2014)
Microtubules	Mouse hippocampus (contextual fear conditioning)	Microtubule stabilizer (paclitaxel) injection immediately after contextual fear conditioning reduces long-term memory. Paclitaxel and microtubule destabilizer (nocodazole) injection 8 h after contextual fear conditioning enhances or reduces long-term memory, respectively.	(Uchida et al., 2014)
Microtubules	Mouse hippocampus (contextual fear conditioning)	Microtubule turnover is changed following contextual fear conditioning. Nocodazole injection reduces contextual fear memory and spine number.	(Fanara et al., 2010)
Microtubules	Mouse (Y maze)	Microtubule stabilizer (epothilone D) treatment rescues deficits in axonal microtubule density and spatial learning observed in aged PS19 mice.	(Zhang et al., 2012)
Microtubules	Mouse (Morris water maze)	Paclitaxel treatment leads to reduced microtubule dynamics and deficits in spatial learning.	(Atarod et al., 2015)

Please note that this is not an exclusive survey and that the findings listed here do not cover all the contents of the reference papers.

Table 2

Brief summary of activity-dependent changes in stathmin phosphorylation in neuronal cells

Stathmin phosphorylation			
Targets	**System**	**Findings**	**References**
Stathmin	Primary neuron	Phosphorylation of stathmin in response to BDNF is inhibited by the specific inhibitor of the MAP kinase cascade.	(Cardinaux et al., 1997)
Stathmin	Primary neuron	Phosphorylation of stathmin is induced by the treatment with dopamine or forskolin.	(Chneiweiss et al., 1992)
Stathmin 2 (SCG10)	Primary neuron	Phosphorylation of SCG10 in response to kainic acid is inhibited by the NMDA receptor antagonist.	(Morii et al., 2006)
Stathmin	Mouse dentate gyrus (contextual fear conditioning)	Phosphorylation of stathmin at Ser16 increases 2-8 h following context-shock exposure. Phosphorylation of stathmin at Ser25 decreases 0.5-1 h following context-shock exposure. Phosphorylation of stathmin at Ser38 decreases 0.5-1 h and increases 8 h following context-shock exposure. Exposure of either context only or immediate shock reduces stathmin phosphorylation at Ser 25 and Ser38.	(Uchida et al., 2014)