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Heterotrimeric G Proteins and the Regulation of Microtubule Assembly

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Abstract

Microtubules (MTs), a major component of cell cytoskeleton, exhibit diverse cellular functions including cell motility, intracellular transport, cell division, and differentiation. These functions of MTs are critically dependent on their ability to polymerize and depolymerize. Although a significant progress has been made in identifying cellular factors that regulate microtubule assembly and dynamics, the role of signal transducing molecules in this process is not well understood. It has been demonstrated that heterotrimeric G proteins, which are components of G protein-coupled receptor (GPCR) signaling pathway, interact with microtubules and play important roles in regulating assembly/dynamics of this cytoskeletal filament. While α subunit of G proteins ($G\alpha$) inhibits microtubule assembly and accelerates microtubule dynamics, $G\beta\gamma$ promotes tubulin polymerization. In this chapter, we review the current status of G-protein modulation of microtubules and cellular and physiological aspects of this regulation. Molecular, biochemical, and cellular methodologies that have been used to advance this field of research are discussed. Emphasis has been given on G-protein-microtubule interaction in neuronal differentiation as significant progress has been made in this field. The outcome from this research reflects the importance of GPCRs in transducing extracellular signals to regulate a variety of microtubule-associated cellular events.

Keywords: cytoskeleton, G-proteins, microtubules, neuronal differentiation, $G\beta\gamma$, tubulin, G protein-coupled receptor, GTP-binding proteins

1. Introduction

The major component of microtubules (MTs) is the heterodimeric protein tubulin, consisting of α and β subunits, which are assembled into linear protofilaments. The protofilaments

associate laterally to form the microtubule, a 25-nm-wide hollow cylindrical polymeric structure [1]. Due to the asymmetry of the $\alpha\beta$ -tubulin heterodimer, MTs are polar structures with two distinct ends. These ends possess different polymerization rates: a slow-growing minus end with an exposed α -tubulin subunit, and a fast-growing plus end, at which the β -tubulin subunit is exposed [2, 3]. MT assembly occurs in two phases: nucleation, which is facilitated by a third tubulin isoform, γ -tubulin; and elongation, during which $\alpha\beta$ -tubulin heterodimers are added to the plus end [1, 4]. Tubulin is a unique guanine nucleotide-binding protein containing one exchangeable binding site and one nonexchangeable binding site. GTP at both sites is needed for optimal assembly, and GTP at the exchangeable site is hydrolyzed after assembly [5, 6]. This hydrolysis creates an MT consisting largely of GDP-tubulin; however, a small region of GTP-bound tubulin, called a “GTP cap,” remains at the end. This cap allows MTs to polymerize. The loss of the cap results in a transition from growth to shortening (called a “catastrophe”), whereas the reacquisition of the GTP cap results in a transition from shortening to growing (called a “rescue”). This behavior, known as dynamic instability, allows MTs to be remodeled rapidly in cells. An important consequence of dynamic instability is that it allows microtubules to search for specific target sites within the cell more effectively [7–9]. The MT assembly process is depicted in **Figure 1**.

MT assembly and stability can be affected by a wide variety of proteins. In this regard, microtubule-associated proteins (MAPs) play a very important role. Members of this group of proteins, such as MAP2 and tau, are known to promote MT assembly and stabilize MTs *in vivo* and *in vitro* [10–13]. The phosphorylation of MAPs is critical for their function, since

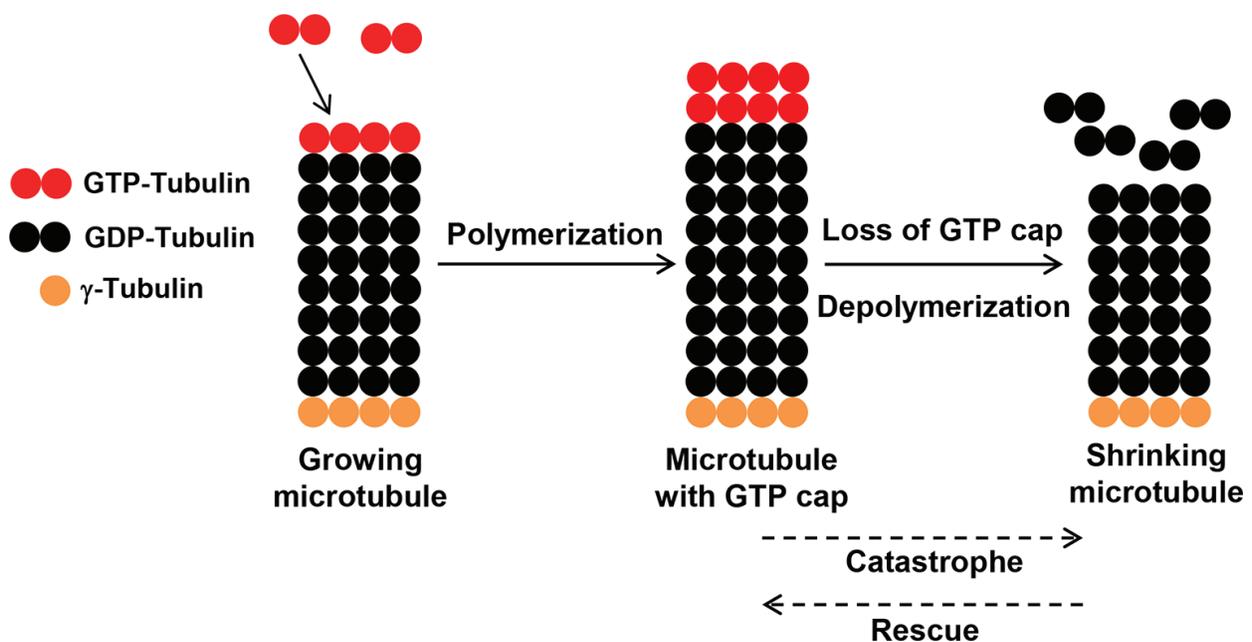


Figure 1. Polymerization/depolymerization of MTs. MTs is polymerized from tubulin heterodimer consisting of α and β subunits. A third tubulin isoform, γ -tubulin, serves as a template for nucleation, which allows proper MT assembly. As shown in the figure, MT assembly requires tubulin to be in GTP bound form. However, it is hydrolyzed to GDP when it is incorporated in MTs, except at the plus (+) end where tubulin remains at GTP bound form (a GTP cap). This cap allows MTs to polymerize. The loss of GTP cap (by hydrolysis) results in the transition to MT depolymerization (a “catastrophe”). GTP cap can be regained by binding to tubulin-GTP and MT polymerization is reestablished (a “rescue”).

phosphorylated MAPs separate from MTs, causing MTs to become more susceptible to disassembly and destabilization [14, 15]. Destabilization of MTs can be promoted by a large number of proteins collectively termed “catastrophe promoters,” as they favor the transition of MTs from elongation to shortening. Examples of these proteins include stathmin/Op18, a small heat-stable protein that is abundant in many types of cancer cells, katanin, and some kinesin-related motor proteins [16, 17]. Also, many drugs known to alter tubulin polymerization are considered valuable tools in studying the mechanisms of MT assembly. Some of these drugs, such as nocodazole, depolymerize MTs, whereas others, such as taxol, promote MT assembly [18–21]. Even though MTs are composed of α/β -tubulin heterodimers in all eukaryotic cells, MTs exhibit great functional diversity. One possible explanation is that both α - and β -tubulin undergo a series of posttranslational modifications that allow MTs to engage in a variety of cellular activities [22]. These modifications include tyrosination/detyrosination, acetylation, glutamylation, and phosphorylation [23]. Although much progress has been made in identifying and characterizing the cellular factors that regulate MT assembly and dynamics, the precise spatial and temporal control of the process is not clearly understood.

Over the past decades, an effort has been made to understand the regulation of MT assembly and dynamics by signal transducing G proteins, as reviewed in Refs. [24, 25]. G proteins are heterotrimer, consisting of guanine nucleotide-binding α plus $\beta\gamma$ subunits. The G-protein-signaling cascade begins with the agonist-induced activation of a G protein-coupled receptor (GPCR), which allows GTP to bind to the α subunit of the heterotrimer, and subsequently, the GTP-bound-activated $G\alpha$ changes its association with $G\beta\gamma$ in a manner that permits both subunits to participate in the regulation of intracellular effector molecules [26]. The traditional pathway for GPCR signaling is shown in **Figure 2**. The GPCR family of proteins is highly diverse; more than 1000 gene-encoding GPCRs are found in the human genome [27, 28]. GPCRs participate in the regulation of a wide variety of physiological functions, including cell growth and differentiation, neurotransmission, immune system function, and hormonal signaling. Participation in such a multitude of processes makes GPCRs a very attractive drug target, and approximately 30% of commercially available drugs are designed to target GPCRs [29]. GPCRs consist of seven transmembrane domains, connected by three extracellular loops and three intracellular loops. The extracellular region is responsible for agonist binding (neurotransmitters, hormones, and odorants, among others), and the intracellular region is responsible for interacting with heterotrimeric G proteins [30]. In humans, there are 21 isoforms of $G\alpha$ subunits, 6 $G\beta$ isoforms, and 12 isoforms of $G\gamma$ [31]. G-protein heterotrimers are typically classified into four classes depending on the $G\alpha$ subunit: $G_{\alpha s}$ (for stimulation of adenylyl cyclase), $G_{\alpha i}$ (for inhibition of adenylyl cyclase), $G_{\alpha q}$ (which regulates phospholipase), and $G_{\alpha 12/13}$, which is involved in the regulation of monomeric G proteins and other molecules, such as PKC [31, 32]. Typical effectors of $G\alpha$ signaling include adenylyl cyclase, phospholipase C, phospholipase A_2 , ion channels, and several kinases and transcription factors. Termination of the signal occurs when GTP bound to the α subunit is hydrolyzed by its intrinsic GTPase activity that causes its functional dissociation from the effector and reassociation with $\beta\gamma$ [26, 33–35]. Thus, G proteins act as molecular switches that can be turned “on” and “off” through the GTPase cycle. While the signal-transducing ability of heterotrimeric G proteins was once believed to depend fully on the α subunit, it has now become clear

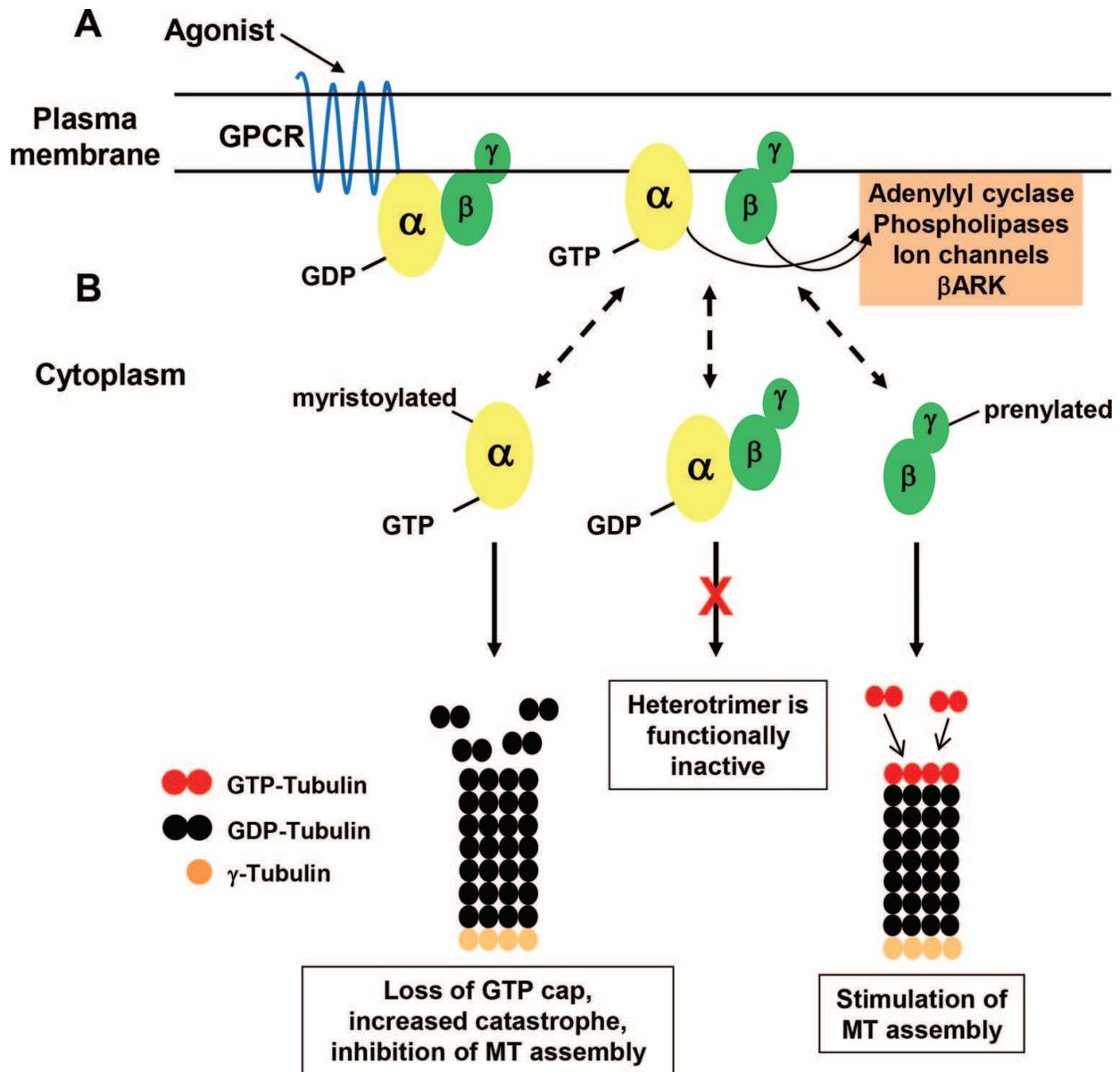


Figure 2. G-protein-mediated signaling and the regulation of MT assembly. (A) The traditional pathway for G-protein signaling cascade begins with the agonist-induced activation of a GPCR (G protein-coupled receptor), which allows GTP to bind to the α subunit of the heterotrimer and subsequently the GTP-bound-activated $G\alpha$ changes its association with $G\beta\gamma$ in a manner that permits both subunits to participate in the regulation of intracellular effector molecules. (B) α and $\beta\gamma$ subunits of heterotrimeric G proteins interact with tubulin/MTs (in cytoplasm) and influence MT assembly and dynamics. Results generated from *in vitro* studies using purified proteins and cultured cells suggest that the $G\alpha$ subunit inhibits MT assembly and promotes MT disassembly by interacting with tubulin-GTP and initiating GTP hydrolysis of tubulin, therefore causing MT depolymerization. The $G\beta\gamma$ subunit, on the other hand, promotes MT assembly. The $G\alpha\beta\gamma$ heterotrimer is functionally inactive (similar to that observed in traditional GPCR pathway) and does not interact functionally with Tubulin/MTs. Upon activation, $G\alpha$ dissociates from $G\beta\gamma$ subunits, and both subunits then interact with tubulin/MTs and modulate assembly/dynamics. It is suggested that G-protein-MT interaction is an important step for G-protein-mediated cell activation.

that the $\beta\gamma$ subunit is capable of interacting with numerous effector molecules to influence a variety of signaling pathways [36, 37]. Among the effector molecules interacting with $G\beta\gamma$ are phospholipases, K^+ and Ca^{2+} channels, GPCR kinases, members of the MAP kinase signaling

pathway, monomeric G proteins, regulators of G protein signaling (RGS), and phosphoinositide-3 kinase (PI3K) [37–42].

Although G proteins are likely to be membrane-bound when coupled to receptors, results from several laboratories in past decades demonstrate their association with several subcellular compartments including MTs. G protein-MT interactions have been shown to modulate the assembly, dynamics and functions of MTs (**Figure 2**). This chapter focuses on our current understanding of G protein regulation of MT assembly and cellular and physiological aspects of this regulation.

2. Heterotrimeric G proteins and the tubulin/MT system

G α and MT assembly/dynamics. Direct interactions between tubulin and α subunits of Gs, Gi1 have been demonstrated [43] and these interactions were shown to activate GTPase activity of tubulin, inhibit microtubule assembly, and accelerate microtubule dynamics [44–47]. To elucidate the role of G α in microtubule assembly *in vitro*, purified G α subunits as well as tubulin were used in the reconstitution assay. G protein α subunits Gi1 α , Gs α , and Go α were shown to activate the GTPase activity of tubulin and inhibit microtubule assembly. The assembly of tubulin-GTP (or tubulin-GppNHp) into microtubules was inhibited by Gi1 α (80–90%) in the absence of exogenous GTP. The addition of exogenous GTP—but not the addition of hydrolysis-resistant GppNHp—overcame the inhibition of microtubule assembly by Gi1 α [45], thus, it appears that GTP hydrolysis resulting from the association of tubulin and Gi1 α plays a critical role in modulating microtubule assembly. G α appears to bind to tubulin and activate the intrinsic GTPase of tubulin in a manner similar to what occurs during MT formation. However, unlike the formation of microtubules from tubulin dimers, G α dissociates from the tubulin-G α complex subsequent to GTP hydrolysis [45]. This finding is consistent with the possibility that G α would accelerate MT dynamic instability. Analysis of the dynamics of individual microtubules by video microscopy has demonstrated that Gi1 α increases the catastrophe frequency [45]. To determine the role of Gs α in MT dynamics *in vivo*, PC12 cells were transfected with Gs α -GFP [47]. Transfected cells were treated with cholera toxin to activate Gs α -GFP or forskolin to stimulate adenylate cyclase and to increase cAMP. Cholera-toxin activation of Gs α -GFP resulted in a displacement of Gs α -GFP from the plasma membrane. It was found that activated Gs α released from the plasma membrane was directly bound to cellular microtubules and then colocalized with microtubules. As a result, activated Gs α made MTs more dynamic, decreasing the pool of insoluble MTs, without changing the total cellular tubulin content [47].

G $\beta\gamma$ and MT assembly. The G $\beta\gamma$ subunit has the opposite effect on tubulin polymerization, as it was found that G $\beta\gamma$ promotes MT assembly *in vitro* [48]. Assembly was monitored by negative staining electron microscopy and measuring protein in polymers collected by centrifugation. The effect of different combinations of $\beta\gamma$ on MT assembly was tested. Tubulin that was purified free of microtubule-associated proteins was incubated at 37°C in the presence of $\beta 1\gamma 2$ or $\beta 1\gamma 1$ (transducin $\beta\gamma$) for 45 min to 1 h. Microtubule assembly was stimulated markedly when $\beta 1\gamma 2$ was present at ~1:20 molar ratio with tubulin; in contrast, $\beta 1\gamma 1$ had no

effect on microtubule assembly [48]. An electron microscopic analysis indicated the formation of very few microtubules either by tubulin alone or in the presence of $\beta 1\gamma 1$. In the presence of $\beta 1\gamma 2$, however, robust microtubule polymerization occurred. Protein estimation in the pellets also indicated a 71% increase in the presence of $\beta 1\gamma 2$. An SDS-PAGE of the samples further confirmed the increase in tubulin concentration in the pellet formed in the presence of $\beta 1\gamma 2$. No detectable change in pellet protein concentration (compared to controls) was observed in the presence of $\beta 1\gamma 1$. β immunoreactivity was detected exclusively in the microtubule fraction after assembly in the presence of $\beta 1\gamma 2$, suggesting a preferential association with microtubules rather than with soluble tubulin. A number of proteins, including the γ subunit of $G\beta\gamma$, undergo a process of posttranslational modification termed “prenylation” and this modification is important for the biological functions of these proteins. For example, prenylation of γ subunits is required for the high-affinity interactions of $G\beta\gamma$ with α subunits or effector molecules [49–51]. Interestingly, it was found that a mutant $\beta 1\gamma 2$, $\beta 1\gamma 2$ (C68S), which does not undergo prenylation of γ subunit, did not stimulate the formation of MTs, suggesting that the functional interaction of $G\beta\gamma$ with MTs require the same specificity as other effector molecules of $G\beta\gamma$ [49–51].

To investigate the potential link between $G\beta\gamma$ and MT assembly *in vivo*, cultured PC12 and NIH3T3 cells were used. The role of $G\beta\gamma$ in MT assembly was demonstrated using nocodazole, a microtubule-depolymerizing drug [52]. Colchicine and the synthetic compound nocodazole are both antimitotic drugs and known to exert their effects by a similar mechanism, that is, by binding to tubulin dimers and inhibiting the subsequent addition of tubulin molecules to microtubules. However, the potential usefulness of nocodazole is due to its readily reversible and rapid activity [53, 54]. Nocodazole-induced depolymerization of microtubules drastically inhibited (~68%) the interaction between $G\beta\gamma$ and tubulin [52]. This result was further confirmed by the isolation of polymerized tubulin (MT) and soluble tubulin (ST) fractions from PC12 cells. Although $G\beta\gamma$ was found in both fractions, a tubulin- $G\beta\gamma$ interaction was found preferentially in MT fractions rather than ST fractions as demonstrated by coimmunoprecipitation analyses. This is consistent with *in vitro* studies, in which $G\beta\gamma$ was preferentially associated with MTs assembled from $\beta 1\gamma 2$ [48]. Removal of nocodazole from the cultured media allowed MTs to repolymerize to their fullest extent and tubulin- $G\beta\gamma$ interaction was restored completely in the MT fraction. These results clearly demonstrate that the association of $G\beta\gamma$ with MTs is important for MT assembly and/or stability. The interactions between $G\beta\gamma$ and tubulin/MTs were also assessed by immunofluorescence microscopy. Microtubules in PC12 cells are well defined and extend to the cell periphery. $G\beta\gamma$ was more concentrated in the perinuclear region where they were colocalized with microtubules. The network of microtubule structure collapsed and $G\beta\gamma$ labeling was dispersed, when cells were treated with nocodazole for 4 h. Microtubules reappeared after the removal of nocodazole, when cells were incubated in fresh media for 4 h. $G\beta\gamma$ labeling was also appeared in perinuclear region where they were colocalized with MTs [52]. In addition to interphase cells, $G\beta\gamma$ -tubulin association was also observed in mitotic spindle in PC12 cells.

$G\beta\gamma$ - γ -tubulin interactions. γ -Tubulin, a member of tubulin superfamily, is a centrosomal protein and its role in MT nucleation is well documented [55–58]. In addition to its binding of $\alpha\beta$ -tubulin, $G\beta\gamma$ was also found to interact with γ -tubulin [33]. However, unlike $\alpha\beta$ -tubulin,

the interaction between γ -tubulin and $G\beta\gamma$ is not inhibited by nocodazole, suggesting that the interaction between $G\beta\gamma$ and γ -tubulin is not dependent upon microtubules [33]. Both $G\beta\gamma$ and γ -tubulin were colocalized in the centrosomes of PC12 cells. Interestingly, γ -tubulin and $G\beta\gamma$ immuno-reactivity appears to be increased significantly in duplicated centrosomes at the onset of mitosis, and $G\beta\gamma$ was consistently found to colocalize with tubulin at mitotic spindle, particularly at the spindle pole areas [33]. Earlier studies in *Caenorhabditis elegans* and *Drosophila* have also demonstrated that $G\beta\gamma$ is involved in cell division by positioning the mitotic spindle and attaching microtubules to the cell cortex [21, 22]. In *C. elegans* embryos, $G\beta\gamma$ was shown to be important in the regulation of migration of the centrosome around the nucleus [21]. These studies collectively suggest an important role of $G\beta\gamma$ in centrosome functions, perhaps through its interactions with γ -tubulin. Although centrosome-associated γ -tubulin is known to be involved in MT nucleation, most γ -tubulin in cells are found in the cytoplasm as γ -tubulin ring complex (γ TuRC) and it has been shown that γ TuRC translocate to centrosome to mediate MT nucleation [59, 60]. Since $G\beta\gamma$ immunoreactivity also increases significantly in duplicated chromosomes at the onset of mitosis [52], it is possible that $G\beta\gamma$ may allow translocation of γ -tubulin to centrosomes. The γ -tubulin- $G\beta\gamma$ complex might then induce robust microtubule nucleation at the centrosome and formation of the mitotic spindle.

$G\alpha\beta\gamma$ heterotrimer and MT assembly. Since G protein activation and subsequent dissociation of α and $\beta\gamma$ subunits are necessary for G proteins to participate in signaling processes [26], it was determined if similar activation is required for modulation of microtubule assembly by G proteins. For that, $G\alpha\beta\gamma$ heterotrimer was reconstituted from α and $\beta\gamma$ subunits and its effect was tested on GTPase activation of tubulin and MT assembly. Myristoylated $Gi1\alpha$ and prenylated $G\beta1\gamma2$ were used to reconstitute the heterotrimer, since lipid modified G-protein subunits have been found to be more effective in interacting with tubulin and subsequent modulation of its functions [45, 48]. In addition, lipid-modified, G-protein subunits have been shown to reconstitute heterotrimers more effectively [61, 62]. Reconstituted heterotrimers have been shown to block $Gi1\alpha$ activation of tubulin GTPase and inhibit the ability of $G\beta1\gamma2$ to promote *in vitro* microtubule assembly [46], suggesting that G-protein activation is required for functional coupling between $G\alpha/G\beta\gamma$ and tubulin/MTs (**Figure 2**). The results also suggest that G protein-coupled receptors (GPCRs) may be involved in the regulation of MT assembly and dynamics *in vivo* by mobilizing G-protein subunits to bind to MTs. In doing so, GPCRs may control a variety of cellular activities. It appears that G-protein-MT interaction is an important step for G-protein-mediated cell activation.

3. G protein-microtubule interactions and cell division

Microtubules play a key role in cell division, participating in the exact organization and function of the spindle apparatus, a vehicle necessary for chromosomal segregation. Microtubules in the spindle are organized in such a way that the minus ends are near the spindle poles, while the plus ends extend toward the cell cortex or chromosomes [63]. Both α and $\beta\gamma$ subunits of G proteins Gi and Go are consistently found to be associated with mitotic spindle. Genetic studies in *C. elegans*, *Drosophila*, and mammalian cells have revealed that G-protein subunits are

involved in regulating mitotic spindle for centrosome/chromosome movements in cell division [64–69]. G-protein α subunits of G_i are involved in cell division by regulating microtubule-pulling forces during chromosomal segregation through a receptor-independent pathway. Unlike the classical G-protein cycle, in which GPCR promotes the GDP/GTP exchange in G_α converting G_α in active GTP-bound form, in nonreceptor pathways, the GDP-bound form of G_α is stabilized through its interaction with guanine-nucleotide dissociation inhibitor (GDI) to regulate microtubule-pulling forces for chromosome movements [66, 70, 71]. Members of the GDI family of proteins, characterized by the presence of 20–25 amino-acid repeats termed “GPR” or “GoLoco” motifs, are known to stabilize the GDP-bound form of G_α by inhibiting the release of nucleotide. Thus, it appears that G_α participates in spindle function through a mechanism that is distinct from the receptor-mediated pathway. In addition to G_α , $G\beta\gamma$ has been shown to play a role in spindle position and orientation during cell division [64, 68]. The association of G_α and β (or $G\beta\gamma$) with spindle microtubules suggests that G-protein subunits may play an important role in the regulation of the assembly and disassembly of mitotic spindles through their ability to modulate microtubule assembly. Recently, it has been shown that reconstituted kinetochores *in vitro* bind preferentially to GTP rather than to GDP microtubules, suggesting that a protein exists in kinetochores that can distinguish between GTP conformation of the microtubules and allow the kinetochores to remain at the microtubule ends to ensure correct chromosome segregation [72]. Since G_α appears to interact preferentially to GTP (rather than the GDP-form of tubulin) and has been detected in mitotic spindles, it may be a likely candidate for segregating chromosomes through its interaction with microtubules.

4. G protein-microtubule interactions and neuronal differentiation

The process by which MT structure is remodeled in neurons is a central question in cell biology and recent research indicates an important role of G protein subunits in this process. During neuronal differentiation, two distinct domains emerge from the cell body: a long, thin axon that transmits signals, and multiple shorter dendrites, which are specialized primarily for receiving signals. The axon terminal contains synapses, specialized structures where neurotransmitters are released to communicate with target neurons. Cytoskeletal structures embodied within neurite extensions and growth cone formations are essential for establishing appropriate synaptic connections and signal transmission. MTs form dense parallel arrays in axons and dendrites that are required for the growth and maintenance of such neurites. In the axon, MTs are bundled by tau, a microtubule-associated protein (MAP), with their plus end oriented toward the nerve terminal. MAP2, a group of high molecular weight MAPs, participates in MT bundling in the dendrites (**Figure 3**). Unlike MTs, actin filaments in neurons are enriched in growth cones and organized into long bundles that form filamentous protrusions, or filopodia, veil-like sheets of branched actin that form lamellipodia [1, 7, 73]. The interaction between these two cytoskeletal filaments is important for the advancement of growth cones and axon guidance [74, 75].

It is clear that cytoskeletal components can detect biochemical signals and respond in order to change the neuronal cell morphology. However, the precise signaling pathways that lead

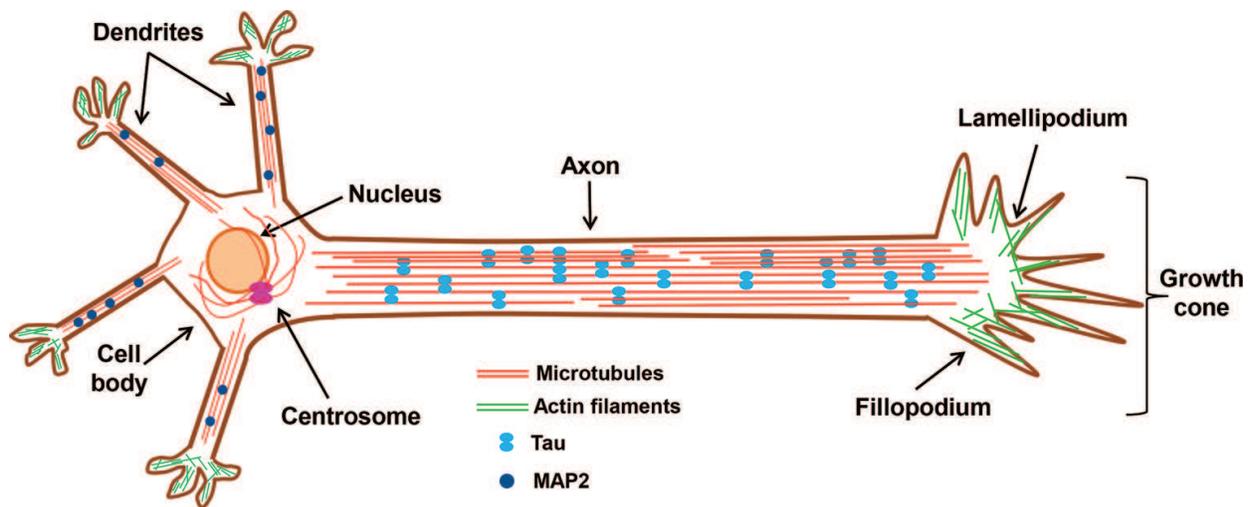


Figure 3. Neuronal cytoskeleton. The polarized and asymmetrical shape of neurons is achieved by means of a highly specialized cytoskeletal organization. In addition to cell body, MTs are found in the axon, dendrites, and the central domain of the growth cone. Tau, a microtubule-associated protein (MAP), participates in MT bundling in the axon, while MAP2 carries this function in the dendrites. Actin filaments are present in the growth cone and dendrites, where they form specialized structures such as lamellipodia and filopodia.

unique organization of MTs in neurons are not clearly understood [76]. PC12 cells have been used extensively for these studies as they respond to nerve growth factor (NGF) with growth arrest and exhibit a typical phenotype of neuronal cells that send out neurites [77]. NGF is a neurotrophic factor critical for the survival and maintenance of sensory and sympathetic neurons. The receptor commonly associated with this process is tyrosine kinase (TrkA) through which NGF exerts its effect [78]. PI3K appears to be the key molecule in this pathway and regulates localized assembly of MTs/actin filaments by downstream Akt/GSK3 β pathways [79, 80]. The Rho and Ras families of small GTPases have also emerged as critical players in regulating the actin and MT cytoskeleton by modulating downstream effectors, including serine/threonine kinase, p21-activated kinase, ROCK, and mDia [81, 82]. GPCRs, as well as α and $\beta\gamma$ subunits of heterotrimeric G proteins, have also been shown to regulate neurite outgrowth [83–90]. These studies collectively suggested the role of α and $\beta\gamma$ subunits of G proteins in regulating neurite outgrowth. More recently, it has been demonstrated that both α and $\beta\gamma$ subunits of G proteins regulate neurite outgrowth and differentiation by interacting with MTs and by modulating MT assembly/dynamics [24].

G α and neuronal differentiation. NGF-induced neuronal differentiation of PC12 cells result in a translocation of G α , Gi1 α , and Go α from cell bodies to cellular processes in which they appear to localize with microtubules [91]. Consistent with this, G α in Neuro-2a cells, which spontaneously differentiate, showed a similar pattern of association of G α with MTs [91]. The result has been further confirmed by transfecting PC12 cells with G α -GFP. Transfected cells were treated with cholera toxin to activate G α -GFP, or forskolin, to stimulate adenylyl cyclase and an increase in cAMP. Colocalization of G α along MTs was seen in cells treated with cholera toxin but not in those treated with forskolin, indicating that activation of G α induces G α translocation to the cytoplasm where it associates with MTs [47]. To understand the function of

Gsα/microtubule association in neuronal development and differentiation, real time trafficking of a *Gsα*-GFP fusion protein was used [92]. GFP-*Gsα* concentrated at the distal end of the neurites in NGF-differentiated living PC12 cells as well as in the cultured hippocampal neurons. *Gsα* appeared to translocate to the growing tip of neurites and to membrane ruffles of the newly formed extensions after NGF treatment, and it has been suggested that during neuronal differentiation, *Gsα* redistributes toward the areas of highly dynamic cytoskeletal activity. Neurite length as well as the number of neurites per cell was also increased in cells overexpressing *Gsα*-GFP in the presence of NGF. The effect was greatest in cells overexpressing constitutively active *Gsα* (*GsQL*). On the other hand, a dominant-negative *Gai*-transducin chimera that interferes with *Gsα* binding to tubulin and activation of tubulin GTPase attenuated the neurite elongation and the neurite number both in PC12 cells and in primary hippocampal neurons. Thus, it appears that activated *Gsα* translocated from plasma membrane induced neurite outgrowth and development through interaction with tubulin/microtubules in the cytosol [92].

Gβγ and neuronal differentiation. The involvement of Gβγ in neuronal development and differentiation has been previously shown [68, 89]. Gβ1-deficient mice have been shown to have neural tube defects [94], and Gβ5-knockout mice have been shown to display abnormal behavior and develop multiple brain abnormalities [95]. It has also been shown that impaired Gβγ signaling promotes neurogenesis in the developing neocortex and increased neuronal differentiation of progenitor cells [68]. Although the mechanism by which Gβγ controls this process is not yet understood, the possibility that Gβγ may act on MTs has been suggested. Sachdev et al. [89] have also suggested that Gβγ-Tctex-1 complex plays a key role in regulating neurite outgrowth in primary hippocampal neurons, most likely by modulating MTs and actin filaments through activation of downstream signaling. These studies suggest a connection between Gβγ signaling and the modulation of MTs during neuronal differentiation and development.

More recently, using biochemical and immunofluorescence analysis, it has been demonstrated that Gβγ-MT interactions and modulation of MT assembly is critical for NGF-induced neuronal differentiation of PC12 cells [94]. To address this, PC12 cells were treated with NGF over the course of three days to allow for neuronal differentiation. Microtubules (MTs) and soluble tubulin (ST) fractions were extracted using a microtubule-stabilizing buffer. The interaction of Gβγ with MT and ST fractions was analyzed by coimmunoprecipitating tubulin-Gβγ complex using a Gβ-specific antibody (rabbit polyclonal anti-Gβ) or a mouse monoclonal anti-α tubulin antibody and determining tubulin and Gβγ immunoreactivity in the complex [94]. Gβγ-MT interaction was significantly increased (2–3 fold) in NGF-treated cells. We also found that MT assembly was stimulated significantly (from 45.3 ± 4.8 to $70.1 \pm 3.6\%$) in NGF-differentiated PC12 cells. The association of Gβγ with MTs in NGF-differentiated cells was also assessed by immunofluorescence microscopy [93]. After NGF treatment, the majority of the cells displayed neurite formation. Gβγ was detected in the neurites and in cell bodies. The colocalization of Gβγ with MTs/tubulin was observed along the neuronal process and in the central portion of the growth cone, but not at the tip of the growth cones.

Overexpression of Gβγ in PC12 cells induced neurite outgrowth in the absence of NGF, further supporting the role of Gβγ in neuronal differentiation [93]. Since Gβ1γ2 promoted

MT assembly *in vitro*—and G β 1 γ 1 had no effect [48], PC12 cells were transfected by either β 1 γ 1 or β 1 γ 2. YFP-tagged β 1, γ 2, or γ 1 constructs were used for transfection. Cells were cotransfected with β 1 and γ 2, or β 1 and γ 1. Within 24 h of transfection, both β 1 γ 1- and β 1 γ 2-transfected PC12 cells were found to overexpress the proteins. At 48 h of transfection, YFP- β 1 γ 2 transfected cells induced neurite formation (in the absence of NGF). Overexpressed protein (YFP-G β 1 γ 2) was localized in the neurite processes, growth cones, and cell bodies. Moreover, overexpressed G β γ exhibited a pattern of association with MTs similar to that observed in NGF-differentiated cells. The average neurite length of G β 1 γ 2 ($42.8 \pm 2.1 \mu\text{m}$) and G β 1 γ 1 ($33.5 \pm 1.8 \mu\text{m}$) is significantly higher than that of control cells ($18.4 \pm 0.6 \mu\text{m}$), with G β 1 γ 2 having the most potent effect on neurite outgrowth. Although the average neurite length in G β γ -overexpressing cells ($42.8 \pm 2.1 \mu\text{m}$) was slightly lower than that observed in NGF-differentiated PC12 cells ($53.6 \pm 1.8 \mu\text{m}$), the result clearly indicates the effectiveness of overexpressed G β γ in inducing neurite outgrowth in the absence of NGF.

Finally, the role of G β γ in neuronal morphology, outgrowth and differentiation was further investigated using peptides and prenylation pathway inhibitors. For example, GRK2i, a G β γ blocking peptide known to inhibit G β γ -dependent effector functions, induced neurite damage as well as MTs and G β γ aggregation. In addition, cellular aggregation was also frequently observed in the presence of GRK2i. The percentage of cell-bearing neurites was reduced significantly. On the other hand, synthetic peptide mSIRK, which is known to activate G β γ signaling in cells by promoting the dissociation of G β γ from α subunits, stimulated neurite formation. Since, γ -subunit of G β γ is known to be posttranslationally modified by prenyl lipid, and prenylation deficient mutant of G β γ (C68S) was shown to be functionally inactive, inhibitors of an enzyme of prenylation pathway (PMPMEase) was tested for their effects on MT assembly and neurite outgrowth. These inhibitors were found to alter MT organization and blocked neurite outgrowth. The results further demonstrate that β γ subunit of heterotrimeric G proteins play a critical role in neurite outgrowth and differentiation by interacting with MTs and regulating MT assembly and organization.

5. Conclusion

Heterotrimeric G proteins transduce signals from cell surface receptors (G protein-coupled receptors) to intracellular effector molecules that include adenylyl cyclase, phospholipases, and ion channels. New evidence suggests that the modulation of the MTs by G proteins is an emerging field of research and therefore an in-depth understanding of G-protein-MTs interaction is important for elucidation of the function, behavior, and morphology of mammalian cells. Key results of this unique interaction may have a broader impact on health and diseases including cancer, Alzheimer's, Parkinson's, depression, and addictive behavior. We foresee that the G-protein-MT dependent pathway could be exploited for developing novel drugs to combat such diseases in the future.

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