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Activation of β - and α_2 - adrenergic receptors stimulate tubulin polymerization and promote the association of $G\beta\gamma$ with microtubules in cultured NIH3T3 cells

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Abstract

Microtubules (MTs) constitute a crucial part of the cytoskeleton and are essential for cell division and differentiation, cell motility, intracellular transport, and cell morphology. Precise regulation of MT assembly and dynamics is essential for the performance of these functions. Although much progress has been made in identifying and characterizing the cellular factors that regulate MT assembly and dynamics, signaling events in this process is not well understood. $G\beta\gamma$, an important component of the G protein-coupled receptor (GPCR) signaling pathway, has been shown to promote MT assembly *in vitro* and in cultured NIH3T3 and PC12 cells. Using the MT depolymerizing agent nocodazole, it has been demonstrated that the association of $G\beta\gamma$ with polymerized tubulin is critical for MT assembly. More recently, $G\beta\gamma$ has been shown to play a key role in NGF-induced neuronal differentiation of PC12 cells through its interaction with tubulin/MTs and modulation of MT assembly. Although NGF is known to exert its effect through tyrosine kinase receptor TrkA, the result suggests a possible involvement of GPCRs in this process. The present study was undertaken to determine whether agonist activation of GPCR utilizes $G\beta\gamma$ to promote MT assembly. We used isoproterenol and UK 14,304, agonists for two different GPCRs (β - and α_2 -adrenergic receptors, respectively) known to activate Gs and Gi respectively, with an opposing effect on production of cAMP. The results demonstrate that the agonist activation of β - and α_2 -adrenergic receptors promotes the association of $G\beta\gamma$ with MTs and stimulates MT assembly in NIH3T3 cells. Interestingly, the effects of these two agonists were

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more prominent when the cellular level of MT assembly was low (30% or less). In contrast to MT assembly, actin polymerization was not affected by isoproterenol or UK 14,304 indicating that the effects of these agonists are limited to MTs. Thus, it appears that, upon cellular demand, GPCRs may utilize $G\beta\gamma$ to promote MT assembly. Stimulation of MT assembly appears to be a novel function of G protein-mediated signaling.

Graphical abstract

Keywords

Microtubules; $G\beta\gamma$; GPCR; Tubulin; Signal transduction

Introduction

The major component of microtubules (MTs) is the heterodimeric protein tubulin. Numerous proteins are known to interact with tubulin and regulate MT assembly either by promoting the polymerization of tubulin or by stabilizing MTs. These proteins are collectively known as microtubule-associated proteins (MAPs). 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* [1, 2]. Several other proteins, including Op18/stathmin, katanin, and some kinesin-related motor proteins, act on MTs by destabilizing the polymer [3–5]. Although these studies identified and characterized the cellular factors that regulate MT assembly and dynamics, the precise spatial and temporal control of the process has not been clearly understood.

Heterotrimeric G proteins play an important role in transferring signals from cell surface receptors (G protein-coupled receptors, GPCRs) to intracellular effector molecules. G protein heterotrimer, consisting of guanine nucleotide binding α plus $\beta\gamma$ subunits, is inactive in cellular signaling. Agonist binding to GPCRs triggers structural rearrangements in the receptors such that the intracellular domain then catalyzes a nucleotide exchange in the α subunit of heterotrimeric G proteins. Subsequently, 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 [6–8]. Although G proteins are likely to be membrane-bound when coupled to receptors, recent results from various laboratories suggest that G proteins associate with MTs, and regulate assembly/dynamics of MTs [reviewed in 9, 10]. It has been

shown that α subunits of G proteins (Gsa, Gi1 α and Gq α) inhibit MT assembly and promote MT dynamics *in vitro* [11]. In contrast, G $\beta\gamma$ promotes MT assembly [12]. Also, reconstituted G $\alpha\beta\gamma$ heterotrimers were found to be inactive in the modulating MT assembly [13], suggesting that receptor-mediated activation of G proteins may involve modulation of MT assembly by G proteins.

To investigate the potential link between G $\beta\gamma$ and MT assembly *in vivo*, cultured NIH3T3 and PC12 cells were used. The role of G $\beta\gamma$ in MT assembly was demonstrated using nocodazole, a MT depolymerizing drug [14]. We found that G $\beta\gamma$ was preferentially bound to MTs in NIH3T3 and PC12 cells and treatment with nocodazole indicated that the dissociation of G $\beta\gamma$ from MTs is an early step in the depolymerization process [14]. When MTs were allowed to recover after the removal of nocodazole, the tubulin-G $\beta\gamma$ interaction was restored in reassembled MTs suggesting that the association of G $\beta\gamma$ with MTs is important for MT assembly and/or stability [14]. More recently, using biochemical and immunofluorescence analysis, it has been demonstrated that G $\beta\gamma$ -MT interactions and modulation of MT assembly is critical for nerve- growth-factor (NGF)-induced neuronal differentiation of PC12 cells [15]. Although, NGF is known to induce neurite outgrowth from PC12 cells by activating the receptor tyrosine kinase, TrkA [16], our study clearly suggests the possible involvement of GPCR since G $\beta\gamma$ is a component of the GPCR pathway.

GPCRs mediate physiological responses to extracellular signaling molecules, such as hormones or neurotransmitters. The GPCR family of proteins is highly diverse; more than 1,000 genes encoding GPCRs are found in the human genome [17]. 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 [6–8]. G-protein heterotrimers are typically classified into four classes: Gs, Gi, GQ and G12-13. Typical effectors of G α /G $\beta\gamma$ signaling include adenylyl cyclase, phospholipases, ion channels, and several kinases and transcription factors [6–8, 18–20]. While activation of the β -adrenergic receptor leads to activation of G α_s and in turn, stimulates adenylyl cyclase and subsequent production of cAMP, activation of the α_2 -adrenergic receptor mediates a decrease in adenylyl cyclase activity and cAMP production through the inhibitory G protein G α_i [7].

The present study was undertaken to determine whether agonist activation of GPCR utilizes G $\beta\gamma$ to promote MT assembly. We found that the activation of β - and α_2 -adrenergic receptors by isoproterenol and UK 14, 304 respectively alters the association of G $\beta\gamma$ with tubulin/MTs and stimulates MT assembly in NIH3T3 cells. This finding may provide a novel mechanism for hormone- or neurotransmitter-mediated alterations of the MT cytoskeleton.

Material and Methods

Cell Culture and Agonist Treatments

NIH3T3 cells (ATCC, Manassas, VA) were grown in 75-cm² culture flasks at 37 °C in DMEM (with 4.5 g/L glucose and L-glutamine), supplemented with 10% bovine calf serum, 0.1 mM sodium pyruvate, and penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) in 10% CO₂. For agonist treatment, cells were grown on 150-mm plates to 80% confluence over 1–2 days and were then treated with 50 µM isoproterenol or 10 µM UK 14,304 (Sigma Chemicals, St. Louis, MO), as indicated in the figures. Stock solutions of 50 mM isoproterenol in deionized water, and 10mM 14,304 in DMSO were prepared, and diluted in culture media to final concentrations of 50 µM isoproterenol and 10 µM UK14,304 respectively. Initial experiments were performed by varying the agonist concentration and the incubation time. A maximal effect was observed when NIH3T3 cells were incubated in the presence of 50 µM isoproterenol or 10µM UK 14,304 for 30-60 min, and therefore was used for subsequent experiments. The similar concentrations (10-50µM) of isoproterenol/UK 14,304 were used in earlier studies showing their effects on cAMP production [7].

Extraction of Microtubules (MT) and Soluble Tubulin (ST) Fractions

NIH3T3 cells were grown on 150-mm plates to 80% confluence over 1–2 days as previously described and subjected to isoproterenol or UK 14,304 as indicated in the figures. The plates were used in duplicate for each condition. Microtubules (MT) and soluble tubulin (ST) were prepared by extracting soluble, and polymerized tubulin fractions in an MT stabilizing buffer (MS) as previously described [14, 15]. Briefly, cells were rinsed in PBS and incubated with 0.5– 1 ml MS buffer (0.1 M PIPES, pH 6.9, 2 M glycerol, 5 mM MgCl₂, 2 mM EGTA, 0.5% Triton X-100) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 1 mM DTT, and 10 µM GTP for ~10 min at room temperature until the cells began to lyse. The cells were removed with a cell scraper and centrifuged for 10 min. The supernatant constitutes the ST fraction and the cell pellets represent the MT fraction. The pellets were resuspended in PEM buffer (100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM MgCl₂) containing 1 mM DTT, 10 µM GTP, and protease inhibitor cocktail followed by incubation in ice for 30 min., sonicated for 20 seconds 3 times and centrifuged for 10 min. Supernatant represents the MT fraction. This procedure yielded highly reproducible results in technically replicated samples used for each condition in a given experiment.

Preparation of Cytosolic Fractions

NIH3T3 cells were grown on 100-mm plates to 80% confluence over 1–2 days. Cells were then treated with or without isoproterenol as indicated in the figure. Incubations were terminated by discarding the media and adding 1 mL ice-cold HEPES-sucrose buffer (15 mM HEPES, 0.25 M sucrose, 1mM DTT, pH 7.5) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 10 min. Subsequently, cells were removed mechanically, homogenized, and centrifuged at 10,000 × g for 10 min. Supernatants represent cytosolic fractions.

Immunoprecipitation

For immunoprecipitation, 100 μ L aliquots of cytosolic fractions (~0.5-1mg/ml) were incubated with and without polyclonal rabbit anti-G γ 2 (Santa Cruz Biotechnology) or non-specific IgG (anti-Gp49) for 1 h at 4 °C, followed by the addition of 100 μ L 50% protein A-sepharose (Amersham Biochemicals, Piscataway, NJ) pre-equilibrated in TBS (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) at 4 °C overnight [14, 15]. Samples were then centrifuged at 12,000 $\times$ g for 6 min in a microcentrifuge, and the supernatants were saved (SUP). The pellets (immunocomplex) were washed with TBST and eluted with 100 μ L 3% SDS Laemmli sample buffer containing 50 mM DTT and boiling in a water bath for 5 min. The samples were then clarified by centrifugation. The supernatant represents the IP and subjected to SDS-PAGE and immunoblotting using α -tubulin-specific antibody as described below.

Electrophoresis and Immunoblotting

Samples for immunoblotting were subjected to SDS-polyacrylamide gel (10%) electrophoresis followed by electro transfer to nitrocellulose membranes. The nitrocellulose membranes were incubated in 5% non-fat dry milk in TBS (10 mM Tris-HCl and 150 mM NaCl, pH 7.4) for 2 h at room temperature, followed by an overnight incubation with monoclonal anti α -tubulin (Sigma) or polyclonal anti-G β or anti-actin (Santa Cruz Biotechnology) specific antibodies (1:250) in 0.01% BSA in TBS. The membranes were washed with 0.05% Tween-20 in TBS (TBST) and incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG in 0.01% BSA in TBST for 1 h. For sensitive detection, a chemiluminescence technique (ECL-plus, Bio-Rad) was used according to manufacturer instructions. A quantitative analysis was done using LabWorks image acquisition and analysis software (UVP Laboratory Products, Upland, CA).

Statistical Analysis

All statistical analyses were performed using Sigma Plot 12 software (Systat Software, Chicago, IL, USA). In the case of the Western blot quantitative analysis and in differences between controls and treatments, the student's paired t-test was employed, and in all cases, a value of $p < 0.05$ was considered to be statistically significant.

Results

Activation of β -adrenergic receptors stimulates tubulin polymerization and increases the association of G $\beta\gamma$ with polymerized tubulin

To determine if receptor-mediated activation of G proteins alters tubulin polymerization (polymer-monomer equilibrium), NIH3T3 cells were treated with isoproterenol, an agonist for β -adrenergic receptors (β -AR). Binding of isoproterenol to the β -AR leads to the activation of Gs heterotrimer (G $\alpha\beta\gamma$), followed by the dissociation of α and $\beta\gamma$ subunits and subsequent activation of adenylyl cyclase [reviewed in 9, 10]. NIH3T3 cells were treated with 50 μ M isoproterenol for 1 h, and MT and soluble tubulin (ST) fractions were prepared by extracting ST in a microtubule-stabilizing buffer (MS) as described previously [14, 15]. The MT assembly was assessed by determining tubulin immunoreactivity in both MT and

ST fractions using anti- α tubulin-specific antibody and measuring the ratio of tubulin incorporated in MTs as a direct measure of MT assembly. We found that MT assembly was stimulated by 37% in the presence of isoproterenol (36.3 ± 6.1 to 49.7 ± 6.5) (Fig.1, A and B). Because previous studies have indicated that G $\beta\gamma$ promotes MT assembly and preferentially associates with MTs both *in vivo* and *in vitro* [12, 14], we performed an immunoblot analysis using anti-G β antibody to test whether the G $\beta\gamma$ association with MTs was altered after isoproterenol treatment. Interestingly, we found that G $\beta\gamma$ association with MTs increased by 57% (32.7 ± 5.5 to 51.5 ± 5) (Fig.1, A and C). Re-probing the blots with anti-actin (Fig.1 A and D) indicated no significant changes in actin polymerization in the presence of isoproterenol.

Further analysis of data obtained from several experiments (six experiments conducted in duplicate) indicates that MT assembly in NIH3T3 cells varied significantly (from 10%–60%) in the experiments conducted. However, the level of polymerized tubulin was highly consistent in technical replicates, indicating the reliability of the methodology. Depending upon the level of polymerized tubulin, MT assembly was modulated by the isoproterenol. As shown in Table 1, a robust increase in MT assembly (124%) was observed in the presence of isoproterenol under conditions in which < 30% tubulin was present in polymerized form. Similar to MT assembly, isoproterenol treatment leads to a robust increase in the association of G $\beta\gamma$ with polymerized tubulin (117%) (Table 1). However, isoproterenol did not alter MT assembly when > 30% polymerized tubulin was present in the NIH3T3 cells (Table 1). Additionally, the association of G $\beta\gamma$ with MTs was not altered under conditions in which MT assembly was unaffected by isoproterenol.

To determine if the tubulin–G $\beta\gamma$ interaction is altered after isoproterenol treatment, cell lysates were prepared and subjected to a co-immunoprecipitation analysis using a G $\gamma 2$ -specific antibody. Anti-G $\gamma 2$ antibody was used for immunoprecipitation since previous studies have demonstrated that G $\beta 1\gamma 2$ promotes MT assembly while G $\beta 1\gamma 1$ showed no effect. The result shows that agonist treatment of NIH3T3 cells induces an increase in the interaction of G $\beta\gamma$ with tubulin (Fig. 2A). In order to test the reliability of the immunoprecipitation results, control experiments were conducted using protein A resins only (without antibody, No ab) and an unrelated rabbit antibody (anti-Gp49). The results show that the anti-G $\gamma 2$ antibody is highly specific (Fig. 2B). In addition, we tested whether isoproterenol increases the expression of G $\beta\gamma$ and tubulin in NIH3T3 cell lysates. The result shows that protein levels remain unchanged after agonist activation (Fig. 2C and D).

Activation of $\alpha 2$ -adrenergic receptors promotes microtubule assembly and leads to the increased association of G $\beta\gamma$ with polymerized tubulin

We also investigated the ability of UK14, 304, an agonist for $\alpha 2$ -adrenergic receptors, to modulate MT assembly in cells. In contrast to isoproterenol, UK14,304 mediates a decrease in adenylyl cyclase activity through the inhibitory G protein, Gi. As shown in the figure (Fig. 3A and B), Gi activation by UK14,304 (10 μ M for 1 h) also stimulated MT assembly by 37% ($36.2 \pm 5.5\%$ to $49 \pm 4.6\%$). Similarly, in the presence of UK14,304, the association of G $\beta\gamma$ with MTs also increased by 74% ($29.5 \pm 5\%$ to $51.4 \pm 4.6\%$) (Fig.3A and C). Re-probing

the blots with anti-actin (Fig.3 A and D) indicated no significant changes in actin polymerization in the presence of isoproterenol.

As observed in the presence of isoproterenol, a robust increase in MT assembly by UK 14,304 (106%) was observed under conditions in which < 30% tubulin was present in a polymerized form (Table 2). No significant change in MT assembly was observed in the presence of UK 14,304 when > 30% tubulin was present as a polymer in cells. The increased MT assembly was correlated with a robust increase (135%) in the association of G $\beta\gamma$ with the MT fraction (Table 2). G $\beta\gamma$ association with MTs remained unaltered under conditions in which MT assembly was unaffected by UK 14,304.

Discussion

The results presented here demonstrate that β - and α 2-adrenergic receptor activation promotes the association/interaction between G $\beta\gamma$ and tubulin/MTs and stimulates MT assembly in NIH3T3 cells. Because β - adrenergic receptors mediate physiological responses through the activation of Gs proteins and subsequent stimulation of cAMP, and α 2-adrenergic receptors activate Gi proteins followed by the inhibition of cAMP [7], the possible involvement of α subunits of G proteins and cAMP/downstream effectors in this process was eliminated. The result indicates that the $\beta\gamma$ subunit of G proteins is likely to be involved in this process. This is further supported by the observation that G $\beta\gamma$ promotes MT assembly *in vitro* and in NIH3T3/PC12 cells [12, 14].

The stimulation of tubulin polymerization by both isoproterenol (agonist for β - adrenergic receptors) and UK 14,304 (agonist for α 2- adrenergic receptors) was dependent on the level of MT assembly and correlated with the increased association of G $\beta\gamma$ with polymerized tubulin fraction. This is supported by previous results from our laboratory indicating that the association/interaction of G $\beta\gamma$ with MT is critical for MT assembly. For example, *in vitro* results indicated that G β immunoreactivity was detected exclusively in the MT fraction after assembly in the presence of $\beta\gamma$ [12]. G $\beta\gamma$ was found to be consistently associated with MTs both in PC12 and NIH3T3 [14]. Furthermore, depolymerization of MTs by nocodazole correlated with decreased association of G $\beta\gamma$ with MTs in PC12 cells, and the association was restored by the removal of nocodazole and subsequent stimulation of MT assembly [24]. The results suggest that when cells require a robust increase in MT assembly, GPCRs may utilize G $\beta\gamma$ to induce MT formation. The observation that pre-incubation of G $\beta\gamma$ with G α (reconstituted heterotrimer) blocked the ability of G $\beta\gamma$ to promote MT assembly [13] suggests that G protein activation is required for functional coupling between G $\beta\gamma$ and MTs and the stimulation of MT assembly. The results presented here using GPCR agonists isoproterenol and UK, 14304 confirm this.

G $\beta\gamma$ has been shown to participate in various MT-dependent processes such as cell growth and differentiation [21–26, 15]. G-protein β -subunit anti-sense oligonucleotides have been shown to inhibit cell proliferation and cause disorganization of the mitotic spindle in mammalian cells [21]. Genetic analyses in *C. elegans*, *Drosophila*, and mammalian cells have revealed that G-protein subunits (α and $\beta\gamma$) are involved in both cell polarity and cell division by positioning the mitotic spindle and attaching microtubules to the cell cortex [22–

24]. The involvement of G $\beta\gamma$ in neuronal development and differentiation has been previously shown [25, 26]. More recently, we have demonstrated that G $\beta\gamma$ -MT interactions and modulation of MT assembly is critical for NGF-induced neuronal differentiation of PC12 cells [15]. Overexpression of G $\beta\gamma$ in PC12 cells induced neurite outgrowth in the absence of NGF, further supporting the critical role of G $\beta\gamma$ -MT interaction in neuronal differentiation [15]. G $\beta 1$ -deficient mice have been shown to have neural tube defects [27], and G $\beta 5$ -knockout mice have been shown to display abnormal behavior and develop multiple brain abnormalities [28]. The results presented here provide a mechanism by which GPCRs regulate various MT-dependent events by mobilizing G $\beta\gamma$ to bind to MTs and stimulate MT assembly and/or stabilization of MTs.

In general, the wide variety of biological functions exerted by MTs (including chromosome movement in cell division, cell differentiation, organelle transport, and the maintenance of cell morphology) is based on the ability of tubulin to polymerize and depolymerize [reviewed in 29]. The data presented here point toward GPCRs as physiological regulators for MT assembly and dynamics. Regulation of MT assembly by G $\beta\gamma$ in response to activation of GPCRs may provide an alternative pathway by which GPCRs function in cellular signaling.

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HIGHLIGHTS

- GPCR activation stimulates microtubule assembly in NIH3T3 cells
- GPCR activation increases G $\beta\gamma$ association with microtubules
- β - and α 2-adrenergic receptors have similar effects on microtubule assembly
- Signaling via GPCR is important for MT assembly and regulation

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Figure 1. Isoproterenol stimulates microtubule assembly and promotes the association of Gβγ with polymerized tubulin

NIH3T3 cells were treated with 50μM isoproterenol for 1 h, and microtubules (MTs) and soluble tubulin (ST) fractions were prepared as described in Methods. Equal amounts of proteins from each fraction were subjected to immunoblotting using anti-tubulin, anti-Gβ, or anti-actin antibodies as indicated. A representative immunoblot is shown from a single experiment (A). Results from protein bands were detected using the ECL-plus reagent, quantitated, and expressed as percent protein in the microtubule (polymerized tubulin) fraction (B, C and D). Values represent the mean ± SE of at least six independent experiments (*p < 0.05).

Figure 2. Co-immunoprecipitation of tubulin with G $\beta\gamma$ using anti-G γ 2 in NIH3T3 cells: the effect of isoproterenol

Cytosolic fractions were prepared as described in Methods from control or isoproterenol-treated cells. Equal amounts of protein were then subjected to immunoprecipitation using anti-G γ 2 antibody. Immuno-precipitates (IP) and supernatants (SUP) were subjected to SDS-PAGE followed by immunoblotting with monoclonal anti- α -tubulin antibody (A). Protein bands were detected and quantitated and are expressed as percent tubulin co-immunoprecipitated with G $\beta\gamma$. The values represent the mean $\pm$ SE of three separate experiments. (B) Cytosolic fractions were immunoprecipitated with an unrelated antibody (anti- Gp49) or no antibody (No ab) as indicated. Cytosolic fractions from isoproterenol treatment were prepared and subjected to SDS-PAGE and immunoblotting using anti-G β (C) or anti- tubulin (D) antibodies.

Figure 3. Activation of α_2 -adrenergic receptors by UK 14,304 promotes microtubule assembly and increases the association of G $\beta\gamma$ with the polymerized tubulin
 NIH3T3 cells were treated with 10 μ M UK 14,304 for 1 h, and soluble and polymerized tubulin samples were prepared. Samples were processed for immunoblotting using anti- α -tubulin, anti-G β , or anti-actin antibodies (A). Protein bands were detected using the ECL-plus reagent, quantitated, and expressed as percent protein in the microtubule (polymerized tubulin) fraction (B, C). Values represent the mean $\pm$ SE of at least five independent experiments (*p 0.05; **p 0.001).

Table 1Isoproterenol effect on modulation of microtubule assembly and the association of G $\beta\gamma$ in MT fraction

Treatment	% Tubulin in MT fraction (MT Assembly)		% G $\beta\gamma$ in MT fraction	
	MT assembly <30%	MT assembly >30%	MT assembly <30%	MT assembly
Control	17.5 $\pm$ 6	51.41 $\pm$ 4.32	26 $\pm$ 6.3	36.66 $\pm$ 1.76
Iso	39.2 $\pm$ 6.2 *	63.2 $\pm$ 6.49	56.5 $\pm$ 6.4 **	46.33 $\pm$ 8.66

*
p=0.01;**
p<0.001

Table 2UK 14,304 effect on modulation of microtubule assembly and the association of G $\beta\gamma$ in MT fraction

Treatment	% Tubulin in MT fraction (MT Assembly)		% G $\beta\gamma$ in MT fraction	
	MT assembly <30% >	MT assembly>30%	MT assembly <30% >	MT assembly >30%
Control	22.1 $\pm$ 6.3	51.41 $\pm$ 4.32	22.8 $\pm$ 3.6	36.67 $\pm$ 1.76
UK	45.6 $\pm$ 6.4 *	59.5 $\pm$ 3.48	53.8 $\pm$ 5.4 **	50 $\pm$ 7.9

*
p=0.01;**
p<0.001