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Tubulin assembly is disordered in a hypogeomagnetic field

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ABSTRACT

Although the effect of a magnetic field on functions of many proteins has been reported, tubulin assembly in a hypogeomagnetic field (HGMF) has not yet been characterized. Here, we show disorder in tubulin self-assembly in an HGMF. Absorbance at 350 nm, commonly used to monitor tubulin self-assembly, was altered in the HGMF, providing evidence for the effects of HGMF on tubulin. Measurements of intrinsic fluorescence (335 nm) also revealed a disordered change in tubulin conformation during assembly in the HGMF. Under the same conditions, microtubule-like filaments were not observed by electron microscopy, with the exception of amorphous oligomers. Incubation of tubulin with tau in the natural geomagnetic field (GMF) yielded microtubule-like filaments, while only amorphous oligomers were observed following the incubation in the HGMF. This distinction suggests that tubulin assembly depends upon the GMF, and that elimination of the GMF induces disorder in tubulin organization.

Numerous reports on the effect of magnetic fields on various cellular processes have been published [1]. The relationship between a biosystem and a magnetic field, especially a strong magnetic field, has been studied by many groups; however, the mechanisms behind the effects of a magnetic field on biomolecules and cells remain unclear.

Recently, the elimination of the geomagnetic field (GMF) was found to interfere with animal brain functions. Long-term memory was impaired in an one-trial passive avoidance task of chicks hatching from an incubator in a hypogeomagnetic field (HGMF) [2]. Wild-type *Drosophila melanogaster* raised in a hypogeomagnetic environment continuously for 10 successive generations gradually becomes impaired in terms of visual conditioning learning and memory formation. Finally, the tenth generation flies become morphs of non-learners and are completely amnesiac [3]. At the level of neurotransmitters, after long-term exposure to an HGMF, both the amount of norepinephrine (NE) and the density of NE-immunopositive neurons in the tissue of golden hamsters decrease significantly, and the effects appear to be progressive with time [4].

Synaptic plasticity, which is crucial for the physical development of animal brains [5], is the ability of a connection, also known as a synapse, between two neurons to change in strength. The circuits in the brain allow an animal to move and experience the world via the senses. It has been demonstrated that a microtubule system is important for the establishment of circuits, through processes such as neurite growth and the formation of connections between axons and dendrites [6]. Microtubules are required for axonal transport and morphogenesis [7,8]. Therefore, tubulin assembly should be paid special attention under conditions in which the GMF is eliminated.

Tubulin is the protein unit of a microtubule. The microtubule is a key cytoskeletal constituent of eukaryotic cells as it is involved in mitosis, cell division, regulation of cellular shape, intracellular transport, and motility [9]. When tubulin molecules assemble in electric or magnetic fields, parallel arrays are formed [10]. Microtubules formed under an electrical field (400 kV/m) show a clear tendency to align along the direction of the electric field [11]. Microtubules have a permanent longitudinal electric dipole [12], which causes them to align parallel to applied electric fields with magnitudes close to the MV/m range. Furthermore, the magnetic field also affects the self-organization of tubulin. Glade and colleagues found that tubulin self-organization is dependent upon the presence of a strong magnetic field [13].

Tau is a major microtubule-associated protein that promotes the assembly and maintenance of microtubules [14]. In a normal neuron, tau is localized to the axon and neuronal soma [15,16]. Thus, the effect of tau protein on tubulin assembly is worthy of study in a weak geomagnetic field. This paper addresses tubulin assembly in the presence and absence of tau in a weak geomagnetic field, and demonstrates disordered tubulin assembly as a result of this treatment.

Abbreviations: HGMF, hypogeomagnetic field; GMF, geomagnetic field

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Materials and methods

Hypogeomagnetic field installation. A compensated HGMF space was produced by three orthogonal Helmholtz coils, each 40 cm in diameter, intersecting one another vertically and thus compensating the values of the geomagnetic field in the three directions along the central axis of each coil: vertical, north to south, and east to west, as described in [3,4]. The magnitude of the residual geomagnetic field resulting from this was 10–100 nT (the natural GMF in this laboratory is about 50000 nT).

Purification of tubulin and tau protein. Tubulin, containing α-/β-subunits, was freshly purified from calf brain after two cycles of assembly and disassembly, as described previously [17]. The tubulin samples were processed through a phosphocellulose column to remove residual microtubule-associated proteins. SDS–PAGE showed that the preparations did not contain any detectable microtubule-associated proteins (Fig. 1a).

A prokaryotic vector (Prk172) bearing the human tau23 gene, a kind gift from Dr. Goedert, was transformed into *Escherichia coli* BL21 (DE3) cells. Recombinant tau protein was produced in these bacteria. Neuronal tau23 was then purified from these bacteria as described in [18]. The purified tau23 protein appeared as a single protein band on SDS–PAGE (Fig. 2a).

Determination of protein concentration. Tubulin was dissolved in 6 M guanidine–HCl, and its absorbance at 275 nm was determined by spectrophotometry. The extinction coefficient for tubulin in guanidine–HCl at 275 nm is 1.03 ml/mg/cm [19]. Tau concentration was measured by a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology Inc., USA).

Tubulin assembly and turbidity measurement. The assembly conditions and the turbidity measurement were performed as described in [20]. Briefly, thawed tubulin was centrifuged (15,000 *g*, 4 °C 10 min) to pellet tubulin aggregates. The supernatant was used in the assembly reaction in the HGMF. Assembly was initiated by the addition of 1 mg/ml tubulin (in the absence of tau) or 0.1 mg/ml tubulin (in the presence of 0.1 mg/ml tau23) to the assembly mixture, which contained 1 mM GTP/1 mM DTT/1 mM $MgCl_2$/1 mM EGTA/100 mM Mes (pH 6.8). The assembly

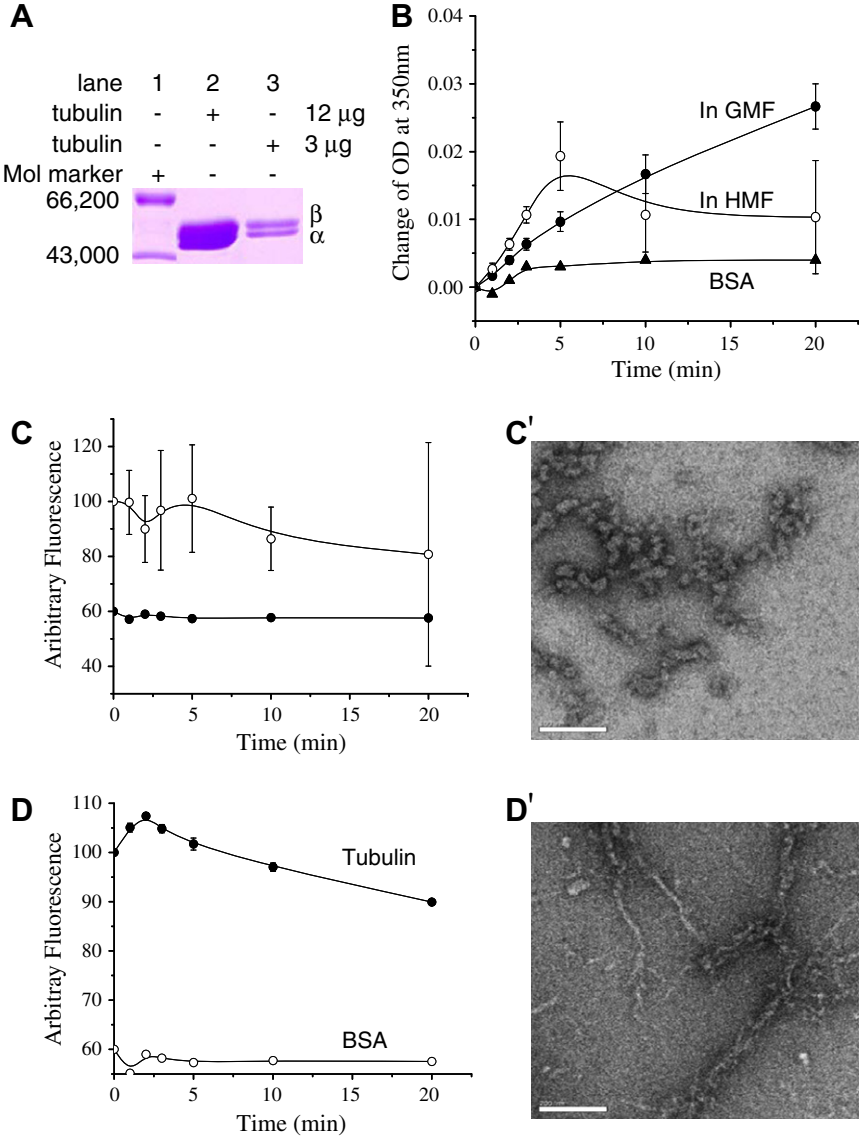

Fig. 1. Tubulin assembly in the HGMF. Tubulin was purified from calf brain as described in [17] (panel a). Tubulin (final concentration 1 mg/ml) was resuspended in 100 mM Mes buffer (pH 6.8) containing 1 mM DTT, 1 mM $MgCl_2$, and 1 mM GTP at 37 °C in the HGMF, as indicated. The absorbance at 350 nm was measured at different time points and changes in the absorbance over time were calculated (panel b). Under the same conditions, the intrinsic fluorescence at 335 nm following excitation at 292 nm was measured in the HGMF (panel c). The fluorescence of a solution of tubulin that underwent self-assembly in the GMF was used as a control (panel d). Tubulin self-assembly in the HGMF (panel c′) and the GMF (panel d′) was analyzed by electron microscopy. Bar = 200 nm.

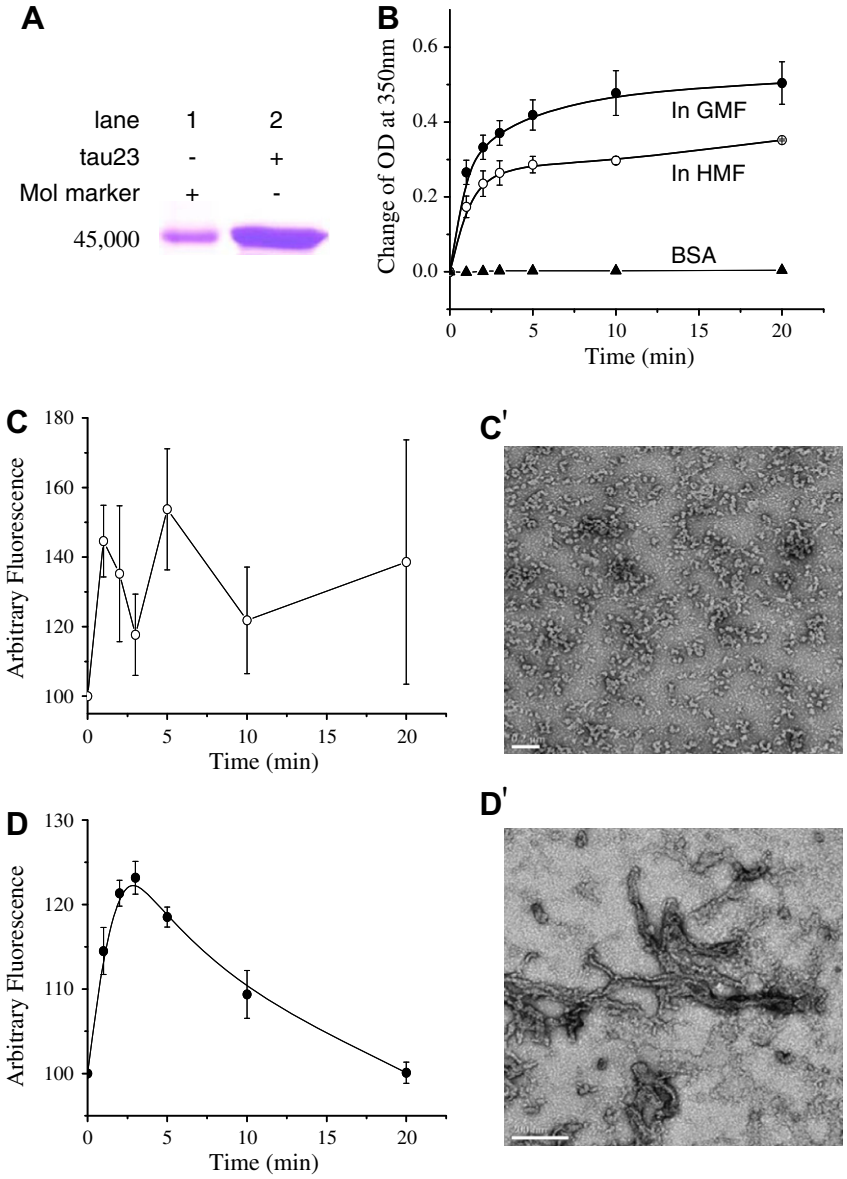

Fig. 2. Tubulin assembly in the presence of tau protein in the HGMF. Recombinant tau23 protein was expressed and purified as described previously [18,27,28] (panel a). Tubulin (final concentration 0.1 mg/ml) and tau (final concentration 0.1 mg/ml) were mixed in 100 mM Mes buffer (pH 6.8) containing 1 mM DTT, 1 mM $MgCl_2$, and 1 mM GTP at 37 °C. Aliquots were taken and submitted the spectrophotometric analysis to determine their absorbance at 350 nm (panel b) and intrinsic fluorescence (panel c) at multiple time points. Tubulin assembly in the presence of tau in the GMF was used as a control (panel d). Electron microscopy was used to observe tubulin assembly in the HGMF (panel c′) and GMF (panel d′). Bar = 200 nm.

process was monitored via absorbance (350 nm) on an F-2010 spectrophotometer (Hitachi, Japan). We utilized the ratio of SD/mean to identify disordered assemblies. SD represents the standard deviation and the "mean" represents the average absorbance value.

Measurement of intrinsic fluorescence. Conditions for tubulin assembly were as described previously [20]. Tubulin was incubated in the presence and absence of tau (37 °C, 30 min) in the HGMF. During the course of tubulin assembly, aliquots were taken at multiple time points to measure the intrinsic fluorescence (335 nm) by excitation at 292 nm on an F-4500 fluorescent spectrophotometer (Hitachi, Japan) [21].

Determination of the critical concentration for tubulin assembly. The desired concentration of tau was 0.8 μM. The assembly buffer and the turbidity measurement were the same as those described above. At least four different concentrations of tubulin were applied in each assembly group. The analysis and the critical concentration determination were performed as described in [19,22].

Sedimentation assay. Tubulin was incubated in Mes buffer (pH 6.8), allowed to stand in the HGMF at 37 °C for 30 min, and then centrifuged (100,000 *g*, 25 °C, 30 min). The pellet was resuspended in the same Mes buffer and then boiled (5 min) prior to submission to SDS–PAGE. Samples treated in parallel in the GMF were used as controls.

Electron microscopy. The assembled microtubules were diluted to 0.1–0.3 mg/ml in Mes buffer supplemented with 30% (v/v) glycerol and 1% glutaraldehyde at 37 °C. The samples (5 μl) were then immediately placed on a formavar-coated EM grid, and incubated (25 °C) for 1 min. The liquid was subsequently wicked-off with filter paper. The samples were washed twice with ddH_2O (10 μl each) at 25 °C, and once with 3% uranyl acetate (10 μl, 1 min) and wicked off again at 25 °C. Finally, samples were visualized using a JEM-100CX electron microscope (JEOL Ltd., Japan).

Results

Disturbance of tubulin self-assembly in the HGMF

To observe tubulin self-assembly, we incubated tubulin in the HGMF and measured the absorbance at 350 nm at multiple time points (Fig. 1b). Changes in the absorbance of tubulin solutions became discrete after the 5 min incubation. The data deviation increased with the length of the incubation. The maximum ratio of SD/mean in the HGMF was as high as 0.8083 after 20 min of incubation, whereas the ratio was only 0.1249 in the natural GMF. The negative control, BSA, showed little change in turbidity. These data suggest that tubulin self-assembly is disturbed in the HGMF.

To further investigate the hypogeomagnetic effect, we measured intrinsic fluorescence during tubulin self-assembly. Changes in the intrinsic fluorescence of tubulin incubated in the HGMF were also discrete (Fig. 1c). The maximum SD/mean ratio was as high as 0.5039. In the local GMF, however, the emission intensity at 335 nm increased at the initial stage (at 2 min) and then decreased with an SD/mean ratio of only 0.0046 (Fig. 1d). This indicates disorder in the self-assembly of tubulin in the HGMF.

Under an electron microscope, tubulin molecules were seen to be present in amorphous oligomers and little microtubule-like filaments could be observed in the HGMF (Fig. 1c′). The size of the oligomers was 27.47 ± 1.96 nm while that of native tubulin was 25.14 ± 1.47 nm. Tubulin self-assembly products in the GMF, which served as a control, appeared as microtubule-like filaments (Fig. 1d′). Their diameter was 26.67 ± 1.13 nm. These data demonstrate that a significant disturbance of tubulin self-assembly occurred in the HGMF.

Tau protein could not restore tubulin self-assembly in the HGMF

As a microtubule-associated protein, tau protein may be able to restore tubulin self-assembly in the HGMF. Absorbance at 350 nm was used to monitor tubulin assembly in the presence of tau23 (Fig. 2b). Changes in turbidity became regular in the presence of tau despite the influence of the HGMF. Tubulin co-incubated with tau in the GMF was used as a control; it showed an ordered increase in turbidity. The data suggest that tau protein may restore tubulin self-assembly in the HGMF.

The intrinsic fluorescence was measured after the addition of tau (Fig. 2c). The fluorescent intensity randomly changed with incubation time and the SD/mean ratio was as great as 0.2535 after a 20 min incubation. Co-incubation of tubulin and tau in the GMF was used as a control; this solution exhibited a regular change in intrinsic fluorescence (Fig. 2d) with an SD/mean ratio of only 0.0125. These data suggest that tubulin assembly in the presence of tau is disordered in the HGMF.

Electron microscopy was employed to investigate tubulin assembly in the presence of tau. Remarkably, amorphous tubulin oligomers were observed in the HGMF (Fig. 2c′). The size of the oligomer granules was 56.00 ± 5.31 nm (greater than that in the absence of tau). Tubulin assembly in the presence of tau in the natural GMF resulted in the generation of microtubule-like filaments (Fig. 2d′) with a diameter of 29.52 ± 2.32 nm.

Characterization of tubulin self-assembly in the HGMF

A sedimentation assay was used to separate the assembled and disassembled tubulin. The pellet and supernatant were both subjected to SDS–PAGE (Fig. 3a). Almost all of the tubulin (~95%) assembled in the GMF (Fig. 3b), while much less of the tubulin assembled (~64%) in the HGMF.

The relationship between tubulin assembly and the initial tubulin concentration was checked during the incubation in both the GMF and the HGMF (Fig. 3c). The minimum concentration required to initiate tubulin assembly was ~1.02 mg/ml in the HGMF, which was higher than that in the GMF (0.84 mg/ml). In contrast, in the

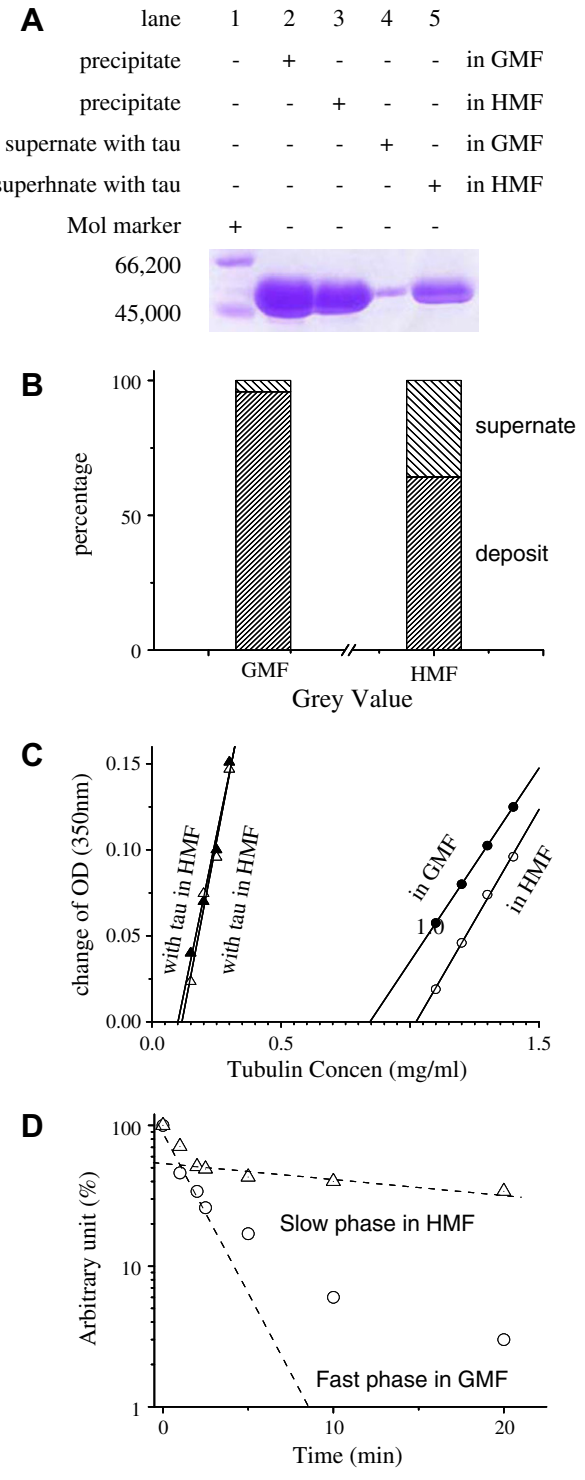

Fig. 3. The critical concentrations for tubulin assembly in the HGMF and GMF. Tubulin was incubated in the HGMF (37 °C, 30 min) and centrifuged (100,000*g*, 25 °C, 30 min). Aliquots of the supernatant and pellet were taken for SDS–PAGE (panel a). Grey density was scanned to show the protein quantity present in the supernatant and pellet, as indicated (panel b). Different concentrations of tubulin were incubated with or without tau protein in the HGMF and GMF, and then the absorbance was measured (panel c). The time course of turbid changes at 350 nm (the same data in Figs. 1b and 2b) were plotted in a semilogarithmic fashion (panel d), according to Tsou's method [23].

presence of tau, the minimum tubulin concentration was at least one order of magnitude lower in all cases (0.1 mg/ml for HGMF and 0.08 mg/ml for GMF) than that required by the samples in the absence of tau. These data indicate that the critical concentration for the initiation tubulin assembly was decreased in the presence of tau in both the HGMF and the GMF. Tau protein promoted tubulin assembly at a lower initial tubulin concentration (0.1 mg/ml), even though microtubule-like filaments were not observed in the hypogeomagnetic environment.

To compare tubulin assembly in the HGMF with that in the GMF, we analyzed the data presented in Figs. 1b and 2b according to Tsou's method [23]. The turbidity of tubulin solutions in the absence of tau protein underwent a monophasic transition in both the HGMF and GMF (Table 1). The first-order rate ($3.84 \times 10^{-3}\ s^{-1}$) of the increase in the turbidity in the HGMF was about one time greater than that in the GMF ($1.92 \times 10^{-3}\ s^{-1}$). This difference in rate suggested that the formation of amorphous oligomers was faster than that of microtubule-like filaments. However, in the presence of tau, changes in the turbidity became biphasic, involving fast and slow phases (Fig. 3d). The first-order rates of the fast phase of tubulin-assembly with tau in both the HGMF and the GMF were greater than those in the absence of tau. Furthermore, the first-order rate of the slow phase in the presence of tau in the GMF was greater than that in the HGMF. This relationship indicates that tau protein not only improves assembly into microtubule-like filaments in the GMF, but also enhances the formation of amorphous oligomers in the HGMF.

Discussion

Tubulin self-organization is dependent upon the presence of a high magnetic field for a brief critical period early in the process [13]. A strong magnetic field influences the orientation of the centrosome and affects the division of *Xenopus leavis* zygotes [12]. Tubulin assembly and organization are involved in the molecular events during cell division. In this work, we have found that disordered tubulin assembly occurred in a weak GMF. Amorphous oligomers were found in the HGMF.

We monitored tubulin self-assembly using turbidity at 350 nm (Fig. 1b). This parameter is indicative of the size of the protein particle in solution, as described in [20]. The intrinsic fluorescence at 335 nm has been measured in the hypogeomagnetic environment. Changes in the intrinsic fluorescence are related to conformational changes of the protein in solution [24], but not to the size of the polymers. Therefore, changes in the turbidity and the intrinsic fluorescence should be different for measurements made during tubulin assembly (Fig. 2b and c). We have measured the emission fluorescent intensity (335 nm) that is contributed by Trp residues. Tubulin (α-/β-subunit) contains eight Trp residues; each of the two subunits contains four Trp residues (A21, A346, A388, and A407 in α-subunit; B21, B103, B346, and B407 in β-subunit) [25]. Tau protein does not contain any Trp residues. Furthermore, Tyr has a fluorescent intensity ($\lambda_{em} = 305$; $\lambda_{ex} = 280$ nm) that is much weaker than that of Trp. Thus, the intrinsic fluorescence should be the combinatorial emission from the eight Trp residues in tubulin. Changes in the fluorescence intensity indicate conformational changes in tubulin. To avoid interference from Tyr fluorescence, we measured the fluorescence at 335 nm by excitation at 292 nm, as described in [21].

As mentioned above, tubulin was treated with phosphocellulose column chromatography to remove the contaminating microtubule-associated proteins. According to Kravit and colleagues [26], this protocol produces oligomers of tubulin. The size of a tubulin monomer is 5–7 nm and the diameter of a microtubule is ~25 nm [26]. The diameter of tubulin amorphous granules is 27.47 ± 1.96 nm. Thus, the granules are oligomers formed in the HGMF. Furthermore, the diameter of the microtubule-like filaments is 26.67 ± 1.13 nm, which is similar to that of natural microtubules (Fig. 1d′). The diameter of tubulin oligomers formed in the presence of tau is approximately 56.00 ± 5.31 nm greater than that formed in the absence of tau. This distinction suggests that the oligomers formed in the presence of tau protein are different from those that formed without tau in the HGMF.

Mavromatos and coworkers proposed the QED-cavity model of microtubules [12]. This refined model predicts dissipationless energy transfer along such shielded macromolecules at near room temperatures as well as quantum teleportation of states across microtubules and perhaps neurons. They calculated the value of the permanent electric dipole moment of the tubulin molecule. Ramalho and colleagues predicted that microtubules have a permanent longitudinal electric dipole, which causes them to align parallel to applied electric fields with magnitudes close to the MV/m range [11]. According to these viewpoints, the permanent electric dipole should result in formation of a relative stable microtubule structure in the GMF. Upon elimination of the GMF, the interaction between the dipole moment of tubulin and the GMF dissipates, resulting in unstable oligomers. Therefore, tubulin molecules oligomerize into disordered amorphous granules.

Summary

Tubulin assembly is sensitive to a decrease of the GMF, which likely prohibits tubulin assembly into microtubule-like structures. This hypothesis is supported by the following observations: (1) tubulin molecules assemble into microtubule-like filaments in the GMF with or without tau protein; (2) changes in the turbidity of the tubulin solution become disordered in the HGMF; (3) the intrinsic fluorescent intensity of tubulin incubated in the HGMF follows a discrete progression; (4) the electron microscopy data indicate that amorphous oligomers form rather than microtubule-like structures in the HGMF; and (5) the tau protein did not restore the intrinsic fluorescence from discrete changes, nor did it restore tubulin assembly from amorphous structures.

Table 1
The first rate constants of changes in turbidity at 350 nm in the HGMF and GMF

	HGMF		GMF	
Reaction phase	Fast phase	Slow phase	Fast phase	Slow phase
Tubulin alone	3.84	–	1.92	–
Tubulin + tau	6.53	0.26	10.01	2.56

The rate constants are in 10^3. The data are from Figs. 1b and 2b under analysis by Tsou's methods [23].

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